

Tobacco and researchers' rights

Scientists have been wrongly condemned for accepting research sponsorship from the tobacco industry. But there are plenty of ways in which the academic community can be harmed or led astray by cigarette manufacturers.

LIKE schoolchildren caught smoking, two scientists were last week forced to account for themselves before finger-wagging British media. Both had taken research funds from the tobacco industry, whose image as the devil incarnate was recently boosted in the United States by President Bill Clinton, in his classification of tobacco as an addictive drug, and by the courts in damages awarded to a former smoker against Brown and Williamson.

Susan Wonnacott, who works on the effects of nicotine on the brain at the University of Bath's School of Biology and Biochemistry, accepted £100,000 from BAT (British American Tobacco Industries, which are also the owners of Brown and Williamson). Jim Edwardson, head of the Medical Research Council (MRC)'s Neurochemical Pathology Unit in Newcastle upon Tyne received £147,000 from BAT for a study on the potential effect of nicotine on Alzheimer's disease (see page 5). Both say that they accepted the nicotine-tainted money because of a lack of other funds to allow them to continue their research. Wonnacott said she would have preferred not to have taken grants that could be "misconstrued by the public", but otherwise defended her action. Edwardson now says he "regrets" his action as he felt it had raised questions about the impartiality of the MRC whereas this "had to be and absolutely has to be seen to be impartial".

But neither researcher appears to have anything to apologize for. On the contrary, their contracts with BAT provide models for other scientists who collaborate not only with tobacco companies but also with other industrial sponsors or government departments that might be tempted to block or influence publication of research they have funded, or to use it for promotional purposes. Wonnacott's contract with BAT had no strings attached. Better still, Edwardson's contract gave him greater control of the results, containing a gagging clause that prevented BAT from using the results in any form without his permission. Had MRC funded the work, BAT could have used them as it wished.

The significance of that point was illustrated earlier this year, when Philip Morris ran full-page advertisements in European newspapers purporting to show scientific evidence that equated the risks of passive smoking with such innocent activities as "drinking 1 to 2 glasses of whole milk per day" or eating biscuits. The campaign — attacked by many scientists as misleading — was concocted not from research that the company had sponsored, but from figures of "relative risk" that it had extracted from the literature.

The researchers' commendable degree of control over their research results should not be taken for granted. A less circum-spect scientist at the University of California, San Francisco (UCSF), last year submitted a paper to the *Journal of the American Medical Association* showing that a drug produced by Boots was not better than cheaper alternatives — not quite the outcome preferred by Boots, which had sponsored the research (see *Nature* **381**, 4; 1995). The contract gave Boots a veto on all publications. Not surprisingly, it used it.

Critics of the British researchers' action rightly suspect that tobacco companies no more sponsor research for the love of

science than do companies such as Boots. The tobacco industry has a long history of abusing scientific data, and any benefit of the doubt it ever deserved has long expired. Smoke alarms should ring when tobacco companies defend their motivation for funding research as being "out of a sense of corporate responsibility".

But, as the MRC rightly argues, if one blacklists tobacco companies, where does one stop? Should researchers also boycott the Playboy Foundation, which funds research on AIDS and human sexuality, on the grounds that to accept funds would be to condone soft porn? Or should scientists avoid defence research on the grounds that the companies involved sell arms to developing countries?

The responsibility for deciding which research funds to accept — and under what conditions — should be left primarily to the judgement of individual researchers, under guidelines from their employers that take the experience and interests of their institutions into account, but which avoid prohibitive political correctness.

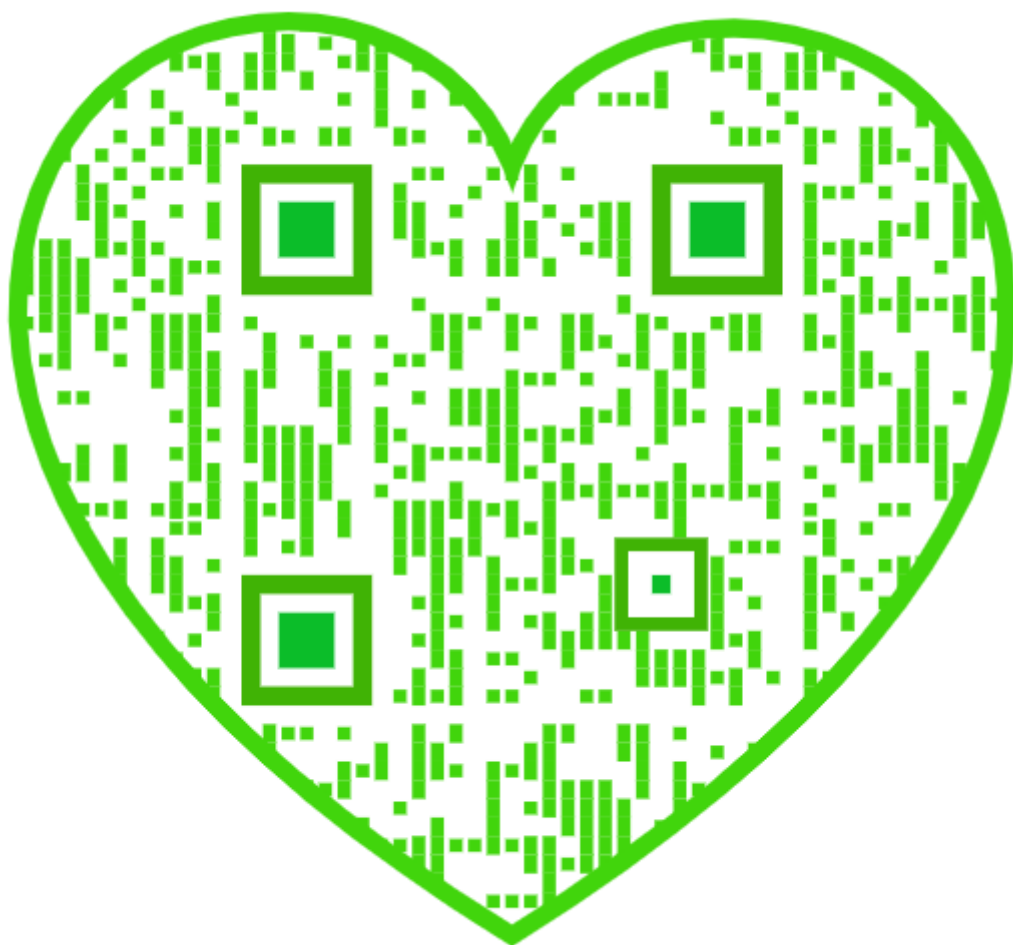
Other forms of sponsorship, where public relations is the prime objective, are more questionable. Take the recent decision by the University of Cambridge to accept £1.5 million from BAT to pay for a chair in its International Studies Department that will be named after BAT's former chairman, Patrick Sheehy. In doing so, the university is celebrating a man whose legacy is to have increased cigarette exports to developing countries.

Cambridge can rightly argue that no brand name is associated with the chair — a Benson and Hedges chair of international studies would have raised a few eyebrows in Cambridge common rooms — but BAT, which earned more than \$25 billion in cigarette sales last year stands to become associated with one of the world's leading universities at a knock-down price.

Cambridge should have rejected the money. So, in a better world, would the scientists who accept the generous prizes sponsored by Philip Morris in many countries — prizes may look good on CVs, but they are hardly essential to science. Accepting such awards is playing into the hands of the cigarette companies, which are increasingly turning to sponsorship because of restrictions on advertising.

But ultimately, the issues raised by the dealings of the scientific community with the tobacco industry are small compared to the threats to public health and science that stem from the industry's political activities. Some US politicians are in the pocket of the tobacco industry, and pose risks to academic freedom. Tobacco-funded legislators have on several occasions tried to block funding of work by Stanton Glantz, a medical researcher at UCSF, who has revealed links between legislators and tobacco interests and made public damaging internal tobacco industry documents (see *Nature* **382**, 6; 1996).

It is a shame that, by taking millions of dollars from tobacco companies, researchers are doing little to weaken the influence of the industry, or its marketing aimed at developing and not-so-developing countries such as China and Japan. The insatiable appetite for tobacco in those populations is providing a new lifeline for the industry and seems impervious to the signals that research is sending. □



Conservationists seek compromise over new threat to Galapagos

Munich. Conservation groups are seeking a last-minute compromise in a bill that has already been approved by Ecuador's National Congress restricting immigration to the Galapagos archipelago, but also allowing a level of economic activity that, they argue, threatens the unique biodiversity of the islands.

The law was drawn up by Eduardo Veliz, a Galapagos congressman, and approved by congress in July. But final approval by Ecuador's new president, Abdala Bucaran, has been delayed while a compromise is sought between the law's supporters and its critics.

The Galapagos islands, which inspired Charles Darwin's theory of evolution, are the world's last remaining oceanic archipelago to preserve most of its original range of individual species, or biodiversity. Ninety-seven per cent of the Galapagos was designated as a national park in the 1960s.

The new law aims to resolve recent conflicts between conservationists and the local population. This has doubled over the last five years as a result of immigration from the mainland, and the population is expected to double again by 2010 if no action is taken.

The high level of conservation has been largely due to the efforts of the Charles Darwin Foundation, which has a research station on the islands, and the Ecuadorian national parks authority, whose stringent rules allow national park land to be used only for tourism and scientific activity.

In the past few years, however, the islands have come under enormous pressure from

immigrants from the mainland seeking a better livelihood in what is now Ecuador's richest province. In addition, the Galapagos marine reserve, which was set up in 1986 to help protect marine resources, is being exploited by fishermen from all over the Pacific, threatening the survival of several species, particularly the sea cucumber, *Isostichopus fuscus* (see *Nature* 373, 465; 1995).

The law drawn up by Veliz sets up a new authority in Galapagos to administer the islands and protect the economic interests of the region and its population, which now stands at 16,000. The Galapagos national park authority will be brought under the jurisdiction of this new authority, but will not be represented on its board.

The Charles Darwin Foundation, which acts as formal adviser to both the national park authority and the government of Ecuador on conservation issues, has welcomed some of the main changes that the law will introduce, particularly the fact that immigration will be restricted.

But it views other changes with concern, in particular the proposed reduction in status of all national parks in Ecuador, allowing "moderate human intervention". In practice this would permit the construction of infrastructure elements, such as roads, to give a boost to local agricultural activity.

Conservationists are also worried about the protection of the marine reserve. Although the law would extend its size by 40 miles from shore, there will no longer be areas designated free from economic exploitation and decisions about fishing

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No safety in numbers: human immigrants threaten Galapagos' unique biodiversity.

quotas will be taken by the government department of fisheries, without reference to conservationists.

The law also specifies that nongovernmental organizations (NGOs) should not be allowed to sell souvenirs. This is intended to allow the local population to benefit economically from tourism. But it would also require the Charles Darwin Research Station, the only NGO in the Galapagos, to close down its own shop, on which it depends for 30 per cent of its income.

Hendrik Hoeck, a former director of the Charles Darwin Research Station, and a member of the Charles Darwin Foundation's council, describes the situation in Galapagos as "very scary". Between 100 and 150 new species of animals and insects have already been introduced by migrants and fishermen, he says, upsetting the ecological balance of the islands.

"This is still the best-managed archipelago in the world, having been able to maintain around 90 per cent of its biodiversity, compared to Hawaii, which has retained only around 40 per cent," says Hoeck. But, he adds, if the current management system is destabilized and conservationists are squeezed out of the system, "the world will lose for ever an important heritage".

Richard Faust, president of the Frankfurt Zoological Society, which is one of the largest independent supporters of the Charles Darwin Foundation, has written to President Bucaran pointing out the dangers in the law, and asking him to consider the social and economic consequences of facilitating loss of biodiversity, which, by stimulating tourism, is the principal source of Galapagos's wealth.

Alison Abbott

African biology federation takes shape

Cape Town. Plans to launch a Federation of Biochemists and Molecular Biologists for Africa are due to be announced during the First Pan-African Conference on Biochemistry and Molecular Biology in Nairobi this week.

According to Peter Campbell, professor of biochemistry and molecular biology at University College, London, the federation is intended to become a sister organization to the European, Pan-American, and Asian and Oceanic federations. Africa has until now been left out, largely because of travel restrictions that accompanied apartheid.

The concept of an African federation has the support of the United Nations Environmental Programme, which has provided facilities for the meeting. In

addition, several non-African scientific societies and funding agencies have provided funds to enable biochemists from all over Africa to attend.

Wieland Gevers, deputy vice-chancellor at the University of Cape Town, and a former president of the Biochemical Society of South Africa, says there is a clear need for the continent's biochemical societies to work together. But he says that achieving this may not be easy in practice because of factors such as the large distances between countries and their serious lack of resources.

He suggests that the viability of an African federation is likely to depend on its receiving financial and organizational support from other bodies, without being dominated by them. **Michael Cherry**

Swedish institute sued for software piracy

Paris. The Karolinska Institute in Stockholm is being sued for software piracy by a Swedish company, Eurodex. The company is seeking SKr10.3 million (\$US1.6 million) in damages, and says it is taking the action because of frustration over the slow progress of a police investigation into the allegations.

This investigation began in June last year, when police, accompanied by Eurodex staff, raided premises of the institute in and around Stockholm. According to Eurodex, a search of 251 computers revealed that 90 contained illegal copies of StatView, a statistics and graphics package that it markets in Sweden for Abacus, a Californian software developer. The company says the software was also found on network servers, although it had not licensed any network versions to the institute.

"We are very surprised that the district attorney has still not made a decision [to prosecute], given that the evidence against the institute is so strong," says Deniz Özen, managing director of Eurodex. One company official says that police involved in the investigation feel that the institute has been "dragging its feet". He also claims the institute was tipped off about the raid, and that police found evidence of attempts to delete

establishing that illegal activity took place is less straightforward than Eurodex claims. The company's estimates of the extent of piracy are based on the discrepancy between the number of software licences returned by researchers (18), and the number of packages found, he says. But not all individuals who bought packages would have filled out and returned the licence forms.

Another complication, says Madstedt, is that under Swedish law he cannot prosecute the institute itself, but must identify and prove the guilt of individuals. (In contrast, the civil action now being taken by Eurodex targets the institute itself and its owner, the Swedish government.)

Madstedt admits, however, that his investigation has taken "much too long". But he says that "the fault is entirely mine", arguing that he has been too busy working on other cases. Madstedt denies reports that he has relied too much on the institute's internal inquiry, although he also agrees that "perhaps the police, and not the institute, should do more now".

Wigzell is also unhappy that Eurodex staff accompanied police during the raids. The police were not computer experts, he says, and relied on Eurodex staff to carry out the computer searches. The raids were therefore "not handled in a clear and objective manner", he says.

Several researchers at the institute also complain that the computers searched could have contained confidential medical information or data with scientific or commercial significance, and are considering starting proceedings to verify that the raid was

carried out in accordance with Sweden's data protection act.

But Daniel Feldman, the president of Abacus, says that the allegations of piracy at the Karolinska institute are only "the tip of the iceberg". A total of 100,000 copies of StatView have been sold worldwide. The company estimates, on the basis of sales of handbooks on the programme, the number of support calls and references to the package in the scientific literature, that more than a million are in circulation.

Feldman is convinced that software piracy is rife in the research community. It lags behind the business world in abiding by copyright laws, he asserts, attributing this to the fact that academic institutions are "not well off", and to a culture of sharing resources. "I think that many institutes need to police this much more; it hasn't been a priority," he says.

One researcher at the Karolinska agrees that the concept of licences is foreign to many researchers: "When you buy a centrifuge it's there for everybody, you don't have licences allowing some people to use it and not others... in this case it happens to be the Karolinska, but it could be anywhere." Since the police raids, "everyone has cleaned up their computers", he says. "We are now catching up with reality."

Other researchers argue that the high price of software adds to the temptation to copy (StatView retails at \$595). Feldman counters that Abacus offers special prices to students (who pay just \$99) and to academic users, and that it is piracy that is keeping the price of software high.

Declan Butler



StatView and other software from computers. "Activity at the institute was very high on the morning of the raid," says the official.

But Hans Wigzell, the vice-chancellor of Karolinska, one of Europe's leading biomedical research institutes, says that it has cooperated fully with police. The case is complex, and its slow pace is therefore not surprising, he adds, pointing out, for example, that many foreign visitors to the institute bring their own software, which they may have licensed at home.

Sweden introduced piracy laws only five years ago, and software copied before and after that date must therefore also be distinguished, he says. An internal inquiry soon to be completed suggests that "nothing [may] come out of the police inquiry", he says.

Kent Madstedt, the regional prosecutor in charge of the investigation, agrees that

Ex-minister ready to share blood blame

Ottawa. In an unusual move, Monique Begin, who was Canada's federal health minister during part of the time when the country's blood supply was infected with HIV and hepatitis C, has voluntarily waived the immunity from blame that has been called for by government lawyers.

Begin, now dean of medicine at the University of Ottawa, has said in a letter to Mr Justice Horace Krever, who is heading a commission of inquiry into the situation: "If you were to have to lay blame, I consider it my duty to take my share of responsibility."

In a recent federal court ruling, Krever won the right to include allegations of wrongdoing against some individuals, but not others, in his final report. The latter group includes 31 former health ministers and numerous senior bureaucrats. But in her letter, Begin says she was shocked to learn that three individuals who had served under her were considered blameworthy, while she and her deputy

and assistant deputy ministers were not.

The three were a former executive director of the Canadian Blood Committee, a director of the Bureau of Biologics and the unpaid chairman of the National Advisory Committee on AIDS, a physician widely admired for his services to AIDS patients. Begin told Krever that these individuals — as well as her department — are blameless and served Canadians "with great professionalism, unquestionable experience and total integrity".

"Justice is offended if people at the top of government in bureaucratic structures are not held responsible for their actions, but employees at less senior levels of the hierarchy are," she said. "Moreover, public ethics requires that those at the top be accountable."

Krever's report is due to be published on 30 September. But he has made it clear that it will not be ready by then because of delays caused by the court challenges to his right to lay blame.

David Spurgeon

Drug and cigarette company donations favour Republicans

Washington. With Republicans seeking to relax regulations on US drug companies — and the Clinton administration to extend controls over tobacco companies — the industries have responded by donating more than three times as much to the Republican Party as to the Democrats in the 18 months that ended in June.

A new study by Common Cause, a Washington group that lobbies for campaign finance reform, shows the Republican Party received \$7.1 million from tobacco and drug companies between January 1995 and June 1996. The Democratic Party received \$2.0 million — or 28 per cent of the Republican figure.

The figures were compiled from reports that have to be filed with the Federal Election Commission. The reports also show that tobacco companies in particular leaned towards the Republicans, donating \$4 million, compared to the \$746,000 they gave to the Democrats.

The donations to the two main US political parties — often referred to as “soft money” — provide a way by which corporate donors and wealthy individuals can by-pass stringent legal limits on campaign contributions. There are no limits on the size of such donations, provided that they help the party generally and no candidate specifically.

Pharmaceutical and tobacco companies in particular have grasped the opportunity to use soft money to court the Republican Party, which took over the US Congress in January last year for the first time in 40 years. For both industries, “Republicans are generally much more sympathetic to their point of view,” says Larry Sabato, a professor of government at the University of Virginia, and co-author of a new book on campaign finance.

And Republicans have not been unresponsive. They have pleased pharmaceutical companies by pushing to amend laws governing the Food and Drug Administration (FDA). Proposed changes would shorten approval times for drugs and medical devices, and relax testing requirements by allowing fewer clinical trials and less data collection by companies.

Tobacco companies, meanwhile, have sought Republican help in fighting what they call an anti-smoking onslaught by the FDA. President Bill Clinton announced last month, for example, that the FDA is to regulate nicotine as an addictive drug, and cigarettes as drug delivery devices. He launched strict new controls on tobacco companies' advertising to children.

Meredith Wadman

Scientists split over tobacco industry research funding

London. Sir Richard Doll, the epidemiologist who first established the link between smoking and lung cancer, last week said he saw “no harm” in tobacco companies funding biomedical research, providing scientists retain “total control” over the use of the funds and the right to publish the research.

Doll was responding to the debate ignited by reports of a decision by the Medical Research Council (MRC) to accept a grant of £147,000 (US\$225,000) from BAT Industries, owners of British American Tobacco. The money is being spent on a three-year study into whether nicotine is of potential benefit to sufferers of Alzheimer's disease.

The MRC's decision has been greeted with astonishment by cancer research charities such as the Imperial Cancer Research Fund, as well as anti-smoking organizations. The outcry has led to an admission from the scientist who accepted the funds that he would not do it again, to spare the MRC the bad publicity. It has also resulted in the temporary suspension of Mary Rice, the MRC's head of public communication, who voiced misgivings in a newspaper interview.

Doll, who is now with the Imperial Cancer Research Fund Cancer Studies Unit at the University of Oxford, describes the reaction as unwarranted. He adds that he has sometimes found industrial sponsors more willing to allow him to retain the right to publish research than government departments, such as the Department of Health, “who have tried to lay down conditions”.

Despite the misgivings of both its public affairs manager and Jim Edwardson, director of its Neurochemical Pathology Unit in Newcastle, the MRC stands by its decision to accept the tobacco company funds.

According to a spokeswoman, BAT has also paid £60,000 for two PhD students at the MRC Toxicology unit at the University of Leicester. Their three-year grant was used for research into the damage to chromosomes caused by a number of chemicals including nitrosodiethylamine, which is found in trace quantities in tobacco smoke.

Jane Lee, the MRC's director of corporate affairs, acknowledges that the question of accepting research funding from tobacco companies is a “grey area” and the subject of debate within the council. But, she adds, a blacklist of unacceptable companies might pose a further dilemma, as other non-tobacco companies engaged in controversial commercial activities would need to be included.

Lee also points out that the conditions attached to the BAT funding are tighter than for some non-tobacco sources. “BAT is not allowed to publicize this research in any way, or use it in promotional

activities, without the MRC's permission.”

The tobacco industry pays £9 billion a year in taxes. Lewis Smith, director of the MRC's Toxicology Unit, says that if people are “willing to go into a hospital paid for by BAT taxes”, he sees no reason why researchers should not use the same source of funds, providing they do not endorse smoking or promote the tobacco company.

But the critics are far from satisfied. Margaret Wilson, head of public relations at the Imperial Cancer Research Fund (ICRF), says the fund was “surprised and disappointed” that the MRC had accepted funds from BAT. Karen Williams, a spokeswoman for the pressure group, Action on Smoking and Health, finds the idea “abhorrent”.

The ICRF collaborates with industry, but would find it “unthinkable” to take money from tobacco companies “which are responsible for a third of all cancer deaths”, says Wilson. Williams questions BAT's motives for giving the money. “There must be something in it for the tobacco industry. The MRC is naive to think there isn't.”

Both Williams and Wilson argue that, while research into the effects of nicotine is essential, it should be funded from outside the tobacco industry. They believe that, despite the MRC's ‘watertight’ conditions, BAT will benefit more from its sponsorship now that the issue has been made public — especially if the research shows nicotine is potentially useful for Alzheimer's sufferers.

Williams says it is “unacceptable” for an industry that is responsible for the hundreds of millions of pounds spent each year fighting nicotine-related cancer to be allowed to benefit from the acclaim that would accompany any positive finding relating to nicotine.

But a spokesman for BAT, which spends £500,000 each year on medical research, says research is funded “out of a sense of corporate responsibility”, and not to obtain publicity, which is why the identity of the recipients is not made public.

He says the funds are allocated by a panel of independent scientists. The research results are “always peer-reviewed and published”, but a decision to acknowledge the source of funds remains with the scientists. Similarly the company cannot use any of the results without the scientists' permission.

One former member of the MRC's governing council suggests that conglomerates that contain tobacco interests ought to give money into a central ‘pool’ of funds that is administered independently. This ought to satisfy the critics and establish the sincerity of the tobacco companies' motives, he says. The BAT spokesman says the idea of pooling funds is “an interesting suggestion worth considering”.

Ehsan Masood

Drug company links test journal's policy

Washington. Readers of the *New England Journal of Medicine* (NEJM) are to be told that a signed leading article in the 29 August issue, favourable to a new anti-obesity drug, was written by two doctors who have acted as paid consultants to the companies marketing the drug.

Marcia Angell, executive editor of the journal, says the editors were not aware at the time that one of the article's authors was linked to Interneuron Pharmaceutical Inc., the company which makes the drug dexfenfluramine, marketed as Redux.

Similarly, she adds, the journal did not know that the second author consults for American Home Products Corporation, which, with Interneuron, markets Redux in the United States, and Servier, the French company that markets it in Europe.

News of the leading article, which was leaked ahead of publication, boosted Interneuron's share price by 13 per cent, to \$33.90. The value of shares in American Home Products rose \$1.50 to \$61.12.

"There was in fact a conflict of interest [in] violation of our policy," says Angell. "We are going to alert our readers as soon as we can." She says the issue will be discussed with the editor-in-chief, Jerome Kassirer, on his return from vacation this week, and the journal will then publish a notification of the conflict of interest.

The case, which has received wide coverage in the US media, has reopened a debate

about the NEJM's strict policy prohibiting since 1990 leading articles by authors who have "ongoing financial associations" with companies that make products discussed in them. Its policy defines "associations" to include "equity interest, regular consultancies, or major research support."

Competitor journals believe the policy, while based on a desirable principle, is undesirable in practice. George Lundberg, for example, editor of the *Journal of the American Medical Association* (JAMA), calls it "unreasonable" because the pool of qualified experts on a topic is often small. Instead of disqualifying authors because of their ties with companies, JAMA merely requires authors of leading articles to disclose such ties. "No other journal in the world" applies the NEJM standard, he says.

Uwe Reinhardt, a professor of health economics at Princeton University who formerly served on the board of NEJM, agrees. Reinhardt, who now sits on JAMA's board, says that, in a world of limited numbers of special experts, "when you're too purist you pay a price".

But Angell disagrees, citing the current article as a case in point. Knowledge that the authors were tied to the manufacturers of the drug being studied will affect a reader's interpretation of the content, she says, and inevitably raises the question: are they biased? "The *New England Journal of Medicine's* point of

view is that no-one should have to wonder."

Nevertheless, Angell says the current situation arose partly out of a "genuine misunderstanding" with the authors, who say they did not consider their arrangements with the drug companies to be regular. Confusion also arose during a telephone conversation in which an NEJM deputy editor is said to have understood from the lead author that both had been consulting for the Food and Drug Administration (FDA), instead of for the companies. Angell says she accepts that the authors did not deliberately intend to deceive the journal or its readers.

The two authors argued that the risk of taking Redux, an appetite-suppressant associated with a rare but fatal lung disorder, "appears to be outweighed by the benefits when the drug is used appropriately". They estimated that 280 lives would be saved by the anti-obesity drug for every 14 people who died from the lung condition, primary pulmonary hypertension.

The authors, JoAnn E. Manson, an associate professor at the Harvard Medical School, and Gerald A. Faich, an adjunct professor at the University of Pennsylvania Medical School, were asked by the NEJM to write the article.

Both say the NEJM policy is ambiguous, as they did not believe that their contact with companies linked to Redux amounted to "regular consultancies". Manson consulted for Interneuron in the autumn of 1995, while the FDA was reviewing the drug. She testified to an FDA panel twice on behalf of Interneuron.

Faich, who began consulting for Servier in 1994, said his "ad hoc" work has consumed less than five per cent of his time in 1996. He testified twice to the FDA on Servier's behalf in the fall of 1995. Faich says he also did 14 hours of work for American Home Products in April, May and June 1996, "on regulatory matters, not on commercial matters". He also worked for the company last month, after the article had been submitted.

"The type of consulting I was doing was by no means 'regular' and it was not 'ongoing'," says Manson. "So there was much room for interpretation of NEJM's policy." She proposed that a disclosure accompany the article, saying that neither author has any financial interest or equity in any drug company producing anti-obesity agents.

Angell says she did not understand from the proposal that the authors had consulted for the drug companies. The suggested wording, while perhaps technically correct, "omit[s] mention of the companies... and consultancy".

There was "no motivation to deceive anyone," says Faich. But "I now of course recognize and deeply regret any problems I've caused the *New England Journal* or its readership."

Meredith Wadman

'Superman' argues for more NIH money

Washington. An emotional speech last week on biomedical research funding to the Democratic National Convention by Christopher Reeve, the 43-year-old actor who once played Superman but who was paralysed from the neck down in a riding accident in 1995, has not gone without response.

Reeve told the delegates in Chicago — as well as a vast national television audience — that he wanted to "challenge" President Bill Clinton. "Our scientists can do more. We've got to give them the chance. And that means more funding for research."

He then cited spinal cord injury as an example of underfunding, arguing that the government spends \$8.7 billion a year caring for victims of such injuries, but only \$40 million on spinal cord injury research. "We've got to be smarter and do better," he concluded.

The next day, Harold Varmus, the director of the National Institutes of

Health (NIH), issued a press release which said that just as spinal cord injury research helps to cure other disorders, so research on diseases such as Parkinson's "is contributing to our understanding of spinal cord injury".

Progress in fighting diseases results from the US government's commitment to research "based on scientific excellence rather than on presumed relevance to certain disorders", said Varmus in the statement.

Henry Bonilla, a Republican congressman from Texas, also took issue with Reeve, claiming that Clinton had sought to reduce the NIH's budget in his first year of office, and emphasizing Republican support for the agency. "Let's set the record straight," said Bonilla. "Ever since then we have had to fight him every year to get [the NIH] the money they need to keep us head and shoulders above any other country." **M. W.**

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Reeve: 'challenge' to
President Clinton.

Japan hints at a shift to the life sciences

Tokyo. Japan's Ministry of Trade and Industry (MITI) and its Science and Technology Agency (STA) are seeking substantial budget increases of 16.9 per cent and 10.7 per cent respectively for 1997. The new money will be used to help finance ambitious new research projects in the life sciences and to strengthen Japan's basic research infrastructure.

Both agencies, together with other ministries, are backing an ambitious brain research programme, and the STA is also seeking ¥1.6 billion for analysing the structure and function of proteins. These are the first indications of the immediate financial impact of Japan's new five-year plan for science and technology, announced in June.

Under the plan, the government has agreed to increase spending on science and technology by 50 per cent over the next five years, with a total spending target for the period of ¥17,000 billion. The requests will probably be pared down by the Ministry of Finance before the budgets are finalized in December. But, despite deteriorating government finances, the government's public promises mean that large increases for science are anticipated.

Ryutaro Hashimoto, Japan's new prime minister, has been calling on government ministries, which can be very territorial and competitive, to increase their cooperation. He is reported to have said that collaborative projects will be given high priority, and senior science officials in Tokyo say that this has led ministries to communicate with each other "very well". MITI wants ¥3 billion for a scheme to collaborate with other ministries in research and development.

The brain research programme is a good example of multiple ministries seeking funds for a multibillion-dollar long-term programme. Various ministries are requesting funding for projects in three major research areas: basic research into the structure and function of the brain; medical research aimed at prevention and treatment of neurological disorders; and research aimed at copying the brain's information-processing functions. The overall brain research plan was sketched out in a document produced by the Science Council of Japan in May (see *Nature* **381**, 266; 1996).

STA has requested ¥10 billion for brain research next year, and ¥7.4 billion to set up a new research institute at the Institute of Physical and Chemical Research (RIKEN), which already has 10 laboratories conducting research related to the brain. Under the plan, the institute will become the focal point of an interministry brain research programme linking national research institutes and universities.

MITI wants ¥2.3 billion for brain research, and plans to set up a new institute — the Brain Function Analysis Experimen-

tal Facility — and to develop research at its National Institute of Bioscience and Human Technology in Tsukuba. The ministry is also seeking funding for collaborative research on brain function for advanced information communication systems with the Ministry of Posts and Telecommunications (MPT). The Ministry of Health and Welfare is also asking for ¥1.5 billion for brain research.

Masao Ito, president of the Science Council of Japan, and one of Japan's leading brain researchers, describes the budget proposals as "a very good start". He hopes the proposed institute at RIKEN, of which he is tipped to become the head, will pull together small and large groups of researchers currently dispersed throughout Japan into a well coordinated cohesive research programme. After the ministries get their various projects up and running, he thinks it will be essential to establish a high-level coordinating committee to manage and direct the overall programme.

In another ambitious project, the STA is asking for almost ten times its 1996 budget for analysing the structure and function of proteins. It has requested ¥1.3 billion to use a beam line at Spring-8, the world's largest synchrotron, due to start operations in Nishi Harima, west of Osaka, next year, to analyse protein structures. The agency is also requesting funding for a new initiative to set up a state-of-the-art nuclear magnetic resonance (NMR) centre at RIKEN (see *Nature* **381**, 105; 1996).

The 'NMR Park', which would be open to foreign researchers, could eventually house more than 50 NMRs and will focus on understanding the fundamental structure of proteins. Akiyoshi Wada, former dean of science at Tokyo University, who heads a group of Japanese chemists and biologists backing the project, believes Japan is well placed to develop new techniques and instruments in the field. He hopes that the new initiative will bring together researchers from various disciplines currently in university departments throughout Japan.

Related to these two projects, the STA is asking for ¥200 million for its National Research Institute for Metals. The money will be used to develop new superconducting magnets to develop the world's most powerful 1GHz NMRs to analyse the structure of

Highlights of Japan's R&D 1997 budget requests in ¥ billion (US\$1 = ¥110)		
	1997	Per cent change
Ministry of International Trade and Industry (MITI)		
Total R&D budget request	338.6	17%
New Sunshine Project	45.6	4%
Intelligent manufacturing systems	1.3	8%
Industrial scientific technology	28.5	8%
Space Flyer Unit	11.3	12%
Nuclear power	24.8	-3%
Global environment research (clean air plan)	17.6	7%
Promotion of R&D for creative industry	4.9	84%
Brain research	2.3	(New)
Joint ministry projects*	3.0	(New)
Science and Technology Agency (STA)		
Total R&D budget request (including extraordinary items)	750.4	8%
General R&D budget request	585.8	11%
Space	183	3%
Nuclear power	324	1%
(Fast breeder reactor research including Monju maintenance)†	93.8	-10%
STA fellowships	3.2	28%
Brain	10	300%
Human genome	3.0	20%
Protein structure analysis	1.8	764%
X-ray laser research	1.4	133%
Spring-8	20.0	20%
Human Frontier Science Programme‡	4.1	20%
* Includes ¥1.0 billion of funds from other ministries.		
† Included in nuclear power budget.		
‡ Includes ¥1.6 billion for MITI.		

enzymes and other large proteins.

STA is seeking to increase its overall funding of the life sciences by 27 per cent next year, but it has only requested a 1.3 per cent increase for nuclear power research. A sodium leak at the fast breeder reactor Monju last December has called into question the future direction of Japan's nuclear programme (see *Nature* **379**, 198; 1996).

Some senior researchers in Tokyo think there could now be a shift towards the life sciences. Japan's international Human Frontier Science Programme, funded by both STA and MITI, has concentrated on brain and molecular function research. Although there is no direct link between it and the newly proposed life science projects, Wada, who expects funding for nuclear power to be flat in the future, suggests that the success of the frontier programme has "implicitly" demonstrated the value of research in these two areas, and has influenced STA officials.

The agency is also hoping to increase its funding of human genome research by 20 per cent, and, almost a year and half after the Kobe earthquake, which killed about 5,000 people, the agency is seeking extra funding for earthquake research.

MITI is seeking extra funds for new schemes to promote venture business and 'creative' industries, and to stimulate research and development at small and medium-sized companies. **Richard Nathan**

Gloom deepens for Australian researchers

Sydney. Australian science has been hit harder than first appeared in last month's budget. A Science and Technology Statement released by the government, covering the seven ministries supporting research and development (R&D), reveals an overall fall of 5.1 per cent in real terms (equivalent to 7 per cent in current-dollar terms), the largest overall drop in funding for many years.

This fall, largely accounted for by a reduction in tax incentives for industrial R&D, is proportionately higher than the budget's 5 per cent cut in operating expenses for universities, spread over three years, which has generated widespread protest (see *Nature* 382, 569 & 659; 1996).

It has also become clear that the Commonwealth Scientific and Industrial Research Organization (CSIRO), Australia's largest research agency, has not escaped unscathed. Government documents reveal that the organization is being required to sell some of its assets to balance an increase of A\$60 million (US\$31.5 million) in operating funds.

CSIRO will also have to repay a so-called 'efficiency dividend', a 2 per cent annual reduction on running costs and 3 per cent on programmes imposed on government activities. The organization is to receive partial compensation of A\$65 million over four years, but will still end up with a net loss of A\$10.3 million.

Two other federally funded agencies — the Australian Nuclear Science and Tech-

nology Organisation, and the Australian Institute of Marine Science (AIMS) — have not only had their operating funds cut but will also not be compensated for losing the 3 per cent efficiency dividend. These agencies will also lose access to their former cash reserves, which had been used as a buffer against unforeseen payments, and had a value equivalent to three weeks' salaries.

Among other cuts, the Defence Science and Technology Organisation has lost A\$20 million (11 per cent) for the year and the Australian Geological Survey Organisation (AGSO) has been slashed by about 25 per cent (see *Nature* 381, 547; 1996). The budget papers had shown an increase for AGSO, but the subsequent science and technology statement revealed that A\$61 million of this is to be spent on a new building.

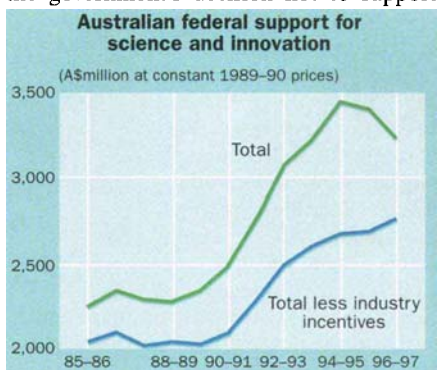
In addition, intense lobbying to reverse the government's decision not to support

Australia's invitation to join the European Southern Observatory (see *Nature* 381, 101; 1996) has been unsuccessful.

A large drop in funding for industrial R&D, in the form of reducing the tax reduction that can be claimed for such research from 150 to 125 per cent, has triggered a chorus of complaints from business, which generally supports the government's cost-cutting strategy. John Prescott, managing director of Australia's largest company, BHP, says that the reduced tax deduction "will mean that some important projects may not go ahead".

Australia, which spends 1.6 per cent of its gross domestic product (GDP) on R&D, lies in twelfth place overall among the 24 leading members of the OECD. But it has long struggled to improve the performance of business R&D, for which it lies in nineteenth place, spending 0.74 per cent of its GDP, compared to an average of 1.11 per cent. Some fear that a steady rise in recent years, said to be due to the 150 per cent tax break, will now be reversed.

Others are also concerned at the likely impact on non-industrial research. "The cuts came at a time when our ageing infrastructure is costing more and more to maintain," says Russell Reichelt, director of the AIMS. The combined effect has caused 20 jobs to go, from total staff of about 180. Areas affected include water quality monitoring on the Great Barrier Reef and large-scale oceanographic work. **Peter Pockley**



Science and Technology Budget Statement, 1996-97

Space centrifuge deal may mean job losses at NASA

Washington. An expected Japanese offer to build a centrifuge for biological research on the international space station has prompted the US National Aeronautics and Space Administration (NASA) to halt its own plans for the facility, a move which may lead to redundancies at the agency's Ames Research Center in California.

In compliance with California's labour laws, about 50 contract employees with the Lockheed Martin Corporation were notified last week that they may be laid off following cuts to the budget for the 1997 fiscal year (which begins on 1 October) for Ames' Space Station Biological Research Project. NASA's space station headquarters at the Johnson Space Center in Houston also told Ames last week to stop any procurements related to the centrifuge.

The deal, if it takes place, would involve Japan building the centrifuge and associated hardware, including an animal-holding facility. In exchange, it would receive a free launch of its Japanese Experiment Module — one of the space station's main laboratories — on the space shuttle in 2000.

Although the details of the barter

arrangement have still to be worked out, Japan is expected to make a formal proposal to NASA by the end of this month. Japanese officials originally suggested a barter deal for launch services last spring, and it was the Americans who suggested that Japan might contribute a centrifuge.

Not having to build the facility itself would solve a short-term financial problem for NASA. Technical difficulties with the construction of one of the station's nodes — small modules that will connect the larger laboratory and living modules — have led to substantial slips in schedule and budget.

According to NASA officials, the cost overruns are likely to be at least \$100 million, and perhaps as much as \$500 million. But the overall space station project is strictly capped at \$2.1 billion per year. Any solution that pushes expenditure to later in the station's development therefore gives NASA some much-needed breathing room.

The centrifuge will be used to provide different levels of gravitation on board the station. It has already had a chequered history. In 1991, the agency proposed eliminating it altogether to save money when

the station was redesigned and scaled back. But the centrifuge was reinstated after scientists complained that its loss would jeopardize the scientific return from the station.

Ames has already spent an estimated \$50 million developing the facility, but has yet to begin construction. Finishing the job would cost an estimated \$100 million, say Ames project managers. It is not clear how much, if any, of the Ames work would be inherited by the Japanese space agency, NASDA, if it takes over responsibility for the centrifuge.

Mary Jane Osborn of the University of Connecticut Health Center, who chairs the National Research Council's Committee on Space Biology and Medicine, says her committee has heard no details about the Japanese proposal, but that it should be judged in terms of how well it serves the research community. Of greater concern, she says, is NASA's apparent backing away from early scientific use of the space station.

Scientists already have been informed that research on the station is being 'rephased' — meaning that it will begin later than the originally planned starting date of November 1998. **Tony Reichhardt**

Research results prompt review of BSE slaughter policy

London. The British government has announced that it is carrying out an immediate review of its cattle-slaughtering plans, following the publication of research results last week that suggest that a selective cull will not eradicate bovine spongiform encephalopathy (BSE) from the British herd.

Using previously confidential statistics on BSE supplied by the Ministry of Agriculture, Roy Anderson, professor of zoology at the University of Oxford, and colleagues concluded that the epidemic is now in decline, and may disappear by 2001 without the need for additional culling (see *Nature* **382**, 777; 1996).

But the researchers concluded that only a prohibitively large slaughter programme would accelerate the elimination of BSE. The Ministry of Agriculture has now announced that its cattle-slaughter programme, which was expected to begin around the end of next month, is to be re-evaluated in the light of the research. □

Cuts 'undermine' space centre

Washington. The head of the Kennedy Space Centre in Florida has warned officials of the National Aeronautics and Space Administration (NASA) that staffing reductions being demanded by Washington could seriously undermine the centre's ability to meet its responsibilities. In particular, wrote Jay Honeycutt in a letter published last week on an Internet site dealing with planned lay-offs at the agency, plans to cut the workforce from its current level of 2,100 to 1,445 in October 1998 would lead to a halt in upgrade work being carried out on the space shuttle after that date. Such plans, he added, could also force the centre to discontinue independent safety studies on shuttle flights, as demanded by the federal commission that investigated the Challenger explosion in 1986. □

European plasma probes launch

Paris. The Interball-2 satellite was launched last week from Plesetsk in northern Russia, along with an accompanying small Czech satellite, Magion-5. They will join Interball-1 and Magion-4, which were launched in August 1995. The Interball project involves 20 countries, including the European Space Agency.

The project is designed to study plasma processes in the atmosphere. It consists of two pairs of satellites, the Tail Probe pair and the Auroral Probe Pair. The former will study solar wind disturbances and X-ray emission bursts on the Sun, while the latter, launched last week, will simultaneously study the impact of these on the magnetosphere and ionosphere. □

Award for scientific journalism

London. Herbert Cerutti of the Swiss daily newspaper *Neue Zürcher Zeitung* has been awarded the 1996 Georg von Holtzbrinck Prize for Scientific Journalism. Cerutti is recognized for "competent and objective scientific reporting of the highest quality". The award was set up by the Georg von Holtzbrinck group, the owner of *Nature*, last year, on the 150th anniversary of *Scientific American*. The DM10,000 (US\$6,750) prize will be awarded in Berlin on 15 November. □

Marine animal centre agreed

Boston. An agreement was reached last month by the International Wildlife Coalition in Falmouth, Massachusetts, to build a National Marine Life Center in Bourne, on Cape Cod, as the world's first facility dedicated to rehabilitating and releasing stranded whales, seals, sea lions, dolphins and turtles.

Joseph Geraci, the president of the centre, says that studying stranded marine animals will not only help them to recover but will

also provide opportunities to learn about the health of animals at sea and of the oceans themselves. Daniel Morast, president of the coalition, says that the centre will help to establish scientifically proven methods for evaluating the condition of the animals washed up on beaches. "Right now our response to stranded animals is well-meaning, but somewhat unscientific," he says. □

Blood scandal scientist arrested

Tokyo. Takeshi Abe, one of the scientists at the centre of the scandal in Japan about the distribution of HIV-contaminated blood products in the mid-1980s, was arrested last week on suspicion of professional negligence resulting in the death of a haemophilia patient.

Abe (pictured right) is the first person to be arrested in the contaminated blood products scandal. In 1983, he was in charge of a Ministry of Health and Welfare AIDS study group that recommended the continued use by haemophiliacs of blood products that had not been heat treated. As a result, nearly half of Japan's 5,000 haemophiliacs were infected with HIV.

Shortly after Abe's arrest, the prosecutor's office raided the Ministry of Health and Welfare. It also raided Nippon Zoki Pharmaceutical Co. Ltd, which distributed Kryobulin, a non-heat-treated blood product which Abe is suspected of administering at Teikyo University Hospital in May and June 1985, when, it is claimed, he should have been aware of the risk of HIV infection. □

Thatcher 'used astrologer'

London. A British newspaper astrologer is claiming to have been approached a decade ago by the former prime minister Margaret Thatcher to keep a watch on her astrological chart and warn of any impending "danger".

Marjorie Orr is reported to have said that she was contacted by Thatcher's press secretary after an IRA bomb killed the wife of one cabinet minister and narrowly missed Thatcher, during a Conservative Party conference in Brighton in 1986. But her former press secretary, Sir Bernard Ingham, has said he could not recall asking Orr to keep a watch on the stars of his boss, a chemistry graduate. He described astrology as "a load of rubbish". □

Human impact assessment

Washington. A three-year multidisciplinary research project to assess the impact of increasing human numbers on plant and animal life will start later this year, sponsored by the American Association for the Advancement of Science. Research at several locations around the world will focus on how factors such as migration, tourism and consumption affect biodiversity. Funded by a \$200,000 grant from the MacArthur Foundation, the project follows a meeting to identify research priorities on biodiversity and population. □

Science festival will be eclipsed

Paris. The organizers of France's annual science festival will this year be praying more earnestly than usual for clear skies. The three-day event will coincide with a partial eclipse of the Sun on 12 October that will be visible for more than two hours across the entire country. "La Science en fête" attracted four million people last year. This year, almost 2,000 events are being staged across France, many at the initiative of local research centres, with about 10,000 scientists taking part. French scientists enjoy a level of respect in society that their colleagues on the other side of the Channel can only dream of. A theatrical representation of the Channel Tunnel itself, "The Victorian connection", will be given in Paris by the Spectrum theatre company from London's Science Museum. □

Chinese scientists drawn back to Asia

The remarkable increase in the number of overseas Chinese scientists returning to the booming economies of Asia is already having an impact on the region. Scientists are also going back to take up key posts in China itself.

THE migration of scientists can have a profound influence on the economic and technological development of nations. Most notably, the impact in the United States of central European emigrants from the 1940s onwards is well documented. But what of the westward migrations of Chinese scientists that have occurred this century? And, of greater potential economic significance, what of the eastward migration of some of those same scientists, and many younger Chinese, that is occurring now?

Waves across the Pacific

Chinese science and engineering students first began to migrate to the West after the Second World War. The preferred destination was the United States, where investment in science was booming. Many of the early migrants from the Chinese mainland came via Taiwan (and a few via Hong Kong), where they and their families had escaped the communist takeover in 1949.

A second wave began in the early 1980s after the renormalization of ties between the United States and China and the re-opening of universities in China following the end of the Cultural Revolution in 1976. Through-

engineering. Fewer than 25,000, or under a quarter, had returned by 1988. A similar number of students and scholars from the Chinese mainland are now in the United States (see page 14).

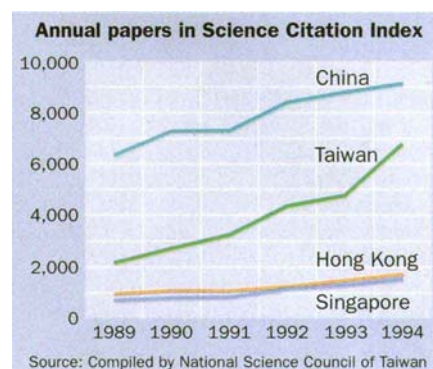
But in the 1990s, a significant return flow began, particularly to Taiwan, where the number of returnees had risen to over 6,000 per year by 1994, about a third of them in science and engineering. Many are senior scientists and engineers with well established careers in the United States who are now taking up positions of leadership in Taiwan. With their Western ways of doing research, these leaders are introducing competition and assessment, so are breaking through the traditional hierarchies that tend to stifle creative research in oriental cultures.

A smaller-scale migration back to Hong Kong and Singapore is also under way: hundreds of Chinese scientists have been drawn back to new universities and institutes, which themselves have been set up by senior Chinese scientists who have returned to the region. Comparatively few are returning to China itself, but those who are going back are taking up key positions.

Those new institutions and returnees have helped boost the output of papers from the region. Papers in *Science Citation Index* for Taiwan, Hong Kong and Singapore more than doubled between 1989 and 1994 (see graph, above right) and the top universities in the region now produce comparable numbers of papers to some of the best universities in the West. China's output has also improved but is still very low on a per capita basis.

The impact on industry has also been profound, particularly in Taiwan, where returnees from Silicon Valley in the United States — where a third of engineers and scientists are said to be of Taiwanese origin — have set up venture businesses in Hsinchu science park for integrated circuits, computers and telecommunications that are competing successfully on the world stage (see page 12).

The returnees and overseas Chinese scientists working in the West are well networked via a number of organizations and are developing research links with colleagues on the Chinese mainland as the research environment there improves with



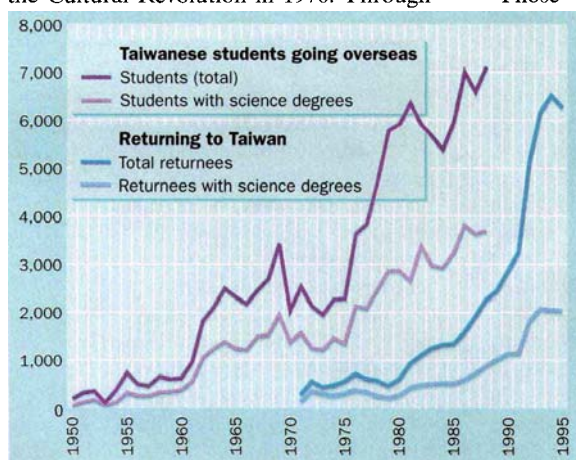
the push for a market economy.

Lure of the tigers

The reasons for Chinese returning to Asia are several-fold. The most important is that economies are booming in the region, and governments in Taiwan, Hong Kong and Singapore are pouring money into creating a strong research and development infrastructure. They firmly believe that they need to develop their own indigenous high technology and can no longer survive on producing existing products with cheap labour, because other less developed nations in the region are more price-competitive.

A prime example of the emphasis on infrastructure comes from Singapore. Through its National Science and Technology Board, the Singapore government has invested S\$2 billion (US\$1.4 billion) over the past five years in new university research institutes and in grants and tax incentives to encourage industry to establish research and development operations. And in Hong Kong, the new Hong Kong University of Science and Technology (HKUST), has grown at breathtaking pace since its establishment (see page 13). But, unlike Singapore, Hong Kong has yet to develop a significant industrial research infrastructure.

The lack of a tradition of pursuing graduate studies in science and engineering in Singapore and Hong Kong has meant that these new institutions tend to recruit overseas, both for academic staff and graduate students. Singapore and Hong Kong are, in a sense, victims of their own success, in ►



The return of science and technology graduates to Taiwan, which has risen markedly since 1990, echoes the earlier migration of students to the West. Data from National Youth Commission and Ministry of Education, Taiwan.

out this period, there was also a steady stream coming from Taiwan to the United States, rising from several hundred per year in the early 1950s to over 7,000 a year in the late 1980s (see graph above).

Between 1950 and the end of the 1980s, more than 110,000 students from Taiwan went overseas, more than 90 per cent to the United States and more than half with undergraduate degrees in science and

On other pages

- Science boost for Taiwan 12
- Research links with China 13
- Why leave the United States? 14
- Singapore taps into talent 14

Taiwan's technocrats stoke the economy

AMONG the rapidly growing number of returnees to Taiwan are many senior people who are taking up top positions in academia and government. They are also powering the development of new high-technology industries. In the 1950s and 1960s, the lack of postgraduate programmes in Taiwan left top graduates who wished to pursue further education with only one option: to go abroad. "At that time 95 per cent of my fellow physics students went to the United States," says Lih J. Chen, professor of materials science and engineering at the National Tsing Hua University, next to Hsinchu science-based industrial park. He returned to Taiwan in 1977 after ten years in the United States and receiving a PhD from the University of California at Berkeley.

Brain drain benefits

But Taiwan is now beginning to benefit from this brain drain as more and more senior researchers and engineers return from the United States and elsewhere. Taiwan's alumni club for Bell Laboratories of the United States boasts more than 200 members and some former returnees are reaching high political office. Taiwan's President Teng-Hui Lee received a PhD from Cornell University in agricultural economics

in 1968, and many of the country's top science officials also have PhDs from universities in the United States.

Over the past few years, the number of senior returnees has reached "a critical mass" that will lead to rapid change, says Winston C. Y. Yu, Secretary General of the National Health Research Institutes, an ambitious new organization, established in January this year, which plans to emulate Britain's Medical Research Council and the US National Institutes of Health (see *Nature* 366, 500; 1993).

The country's rapid economic development and expanding postgraduate programmes have opened up new job opportunities for highly educated people. These, and a strong desire "to help out" and "make a contribution", are often cited as the prime motivation for returning.

The number of graduate students in Taiwan has increased more than tenfold from 3,912 in 1975 to 42,097 in 1995. But Taiwan is still losing thousands of bright students to the United States each year, because living conditions are far from ideal and those in the forefront of research find "the standard in the US really much more exciting", one leading academic says.

The government's National Science

Council (NSC), which has five US offices (including one in Silicon Valley), is intensifying its efforts to encourage more of these students to return. In 1993, it set up a grants programme to help returnees to start their research quickly without competing head-on with researchers already established in Taiwan. A total of 403 researchers was recruited under the programme in 1995, and US\$12 million is allocated to recruit a similar number of "experts" in 1996.

The science-based industrial park in Hsinchu, also administered by the NSC, which has made Taiwan a world leader in the production of personal computers and integrated circuits, is continuing to attract returnees. The park, established in 1980, has 193 companies, 81 set up by returnees. It is already in its third phase of development, and a fourth phase, as well as a second park in Tainan, southern Taiwan, is being planned by the NSC. Sales now exceed \$11 billion a year and the park, where 50,000 people (including 2,400 returnees) are employed, accounts for about 3 per cent of Taiwan's total manufacturing output.

High-technology focus

According to H. Steve Hsieh, vice-chairman of the NSC, returnees with extensive experience in industry in the United States are helping transform Taiwan into "high-tech island" at a crucial time when more and more labour intensive light-industry is moving to the Chinese mainland. US companies and institutes are thus acting like "incubators" for Taiwan, he says.

The park and the nearby Industrial Technology Research Institute (ITRI), funded by the Ministry of Economic Affairs, have provided a huge impetus for Taiwan's booming integrated circuit industry. In fact, the industry is so successful that ITRI is having to search for a new purpose because industry no longer needs its support.

New types of companies are also emerging. In 1993, Peter Kuan, returned from the United States to set up Taiwan's first bio-engineering company in the park, United Orthopedic Corporation. The company, which now employs 56 people, produces orthopedic implants designed especially for orientals and it hopes to export to other Asian markets, including China.

Genelabs Biotechnology is another ambitious new company at the park which hopes to win approval for Taiwan's first domestically-developed drug (most drugs produced in Taiwan are generics of drugs developed in the West). But Hsinchu's attempts to move into biotechnology have not yet been as successful as the integrated circuit industry, partly because top bio-scientists are being attracted elsewhere in Taiwan.

Richard Nathan

Academia Sinica pulls back scientists

ACADEMIA Sinica, Taiwan's leading government research organization, with over 20 research institutes, is a magnet for senior returnees. In 1994, Yuan-Tseh Lee, a Nobel-prize winning chemist from the University of California at Berkeley, returned to take on the presidency of the organization.

Almost all the directors of its 22 institutes have been educated abroad. But the number of senior scientists joining after Lee's appointment is increasing. Shing-Fu Yang, a well known plant physiologist and a member of the US National Academy of Sciences, became director of the Institute of Botany eight months ago, and Kuyung Lu, chairman of the department of astronomy at the University Illinois, will head the academy's new Institute of Astronomy and Astrophysics. Another senior scientist, Kungyu Wo from the University of Houston, is set to become director of the Institute of Medicine and the trend is likely to continue.

The number of papers published by scientists in Taiwan in international journals has been increasing steadily (see

page 11), but Lee thinks it is now essential to pursue quality to establish Taiwan as centre of excellence in the Asia-Pacific region. I want people overseas to "raise eyebrows" when they see what we are doing here, he says.

While connections with academics and institutions in the United States are strong and getting stronger, there is little collaboration between researchers at the academy and the Chinese mainland.

The National Science Council has set aside \$800,000 this year to stimulate links with the mainland. Some researchers are also building their own links. One example is Wean-Shun Tsay and his colleagues at the Institute of Astronomy, National Central University in Taiwan. They are collaborating with the Beijing Astronomical Observatory and astronomers in the United States (many of whom are Chinese) in a multicolour sky survey project entitled the Beijing Arizona Taipei Connecticut Project (BATC), making use of a 24-inch Schmidt telescope at Beijing observatory.

R.N.



Lee: leading the flow back to Taiwan.

► that home-grown graduates in science and engineering often choose to go straight into high-paying local jobs rather than pursue postgraduate research. Singapore is trying to rectify this by topping up incomes for local graduate students to persuade the brightest to stay. 'Accelerated' courses are also offered, to hasten students to a masters degree in the time it takes to get an undergraduate degree.

But it is an "uphill struggle", says Bernard Tan, dean of science at the National University of Singapore (NUS). Because many of the leaders of the new institutions are of Chinese ethnic background, it is only natural that through their connections they recruit Chinese scientists from around the world. It is not that Chinese scientists are being targeted for positions — NUS also recruits many Indian scientists and has a cosmopolitan mix of faculty, as does HKUST. The Chinese are just such a "huge pool" says Tan. Furthermore, "how many non-Chinese want to come to Hong Kong?", points out HKUST president Chia-Wei Woo.

For Taiwan, the situation is rather different. Taiwanese students have a long tradition of pursuing graduate study. For many years this was done in the United States, but graduate schools in Taiwan are now quite strong, with more than 40,000 students (see opposite). Also, in contrast to Singapore and Hong Kong, it is harder for Taiwan to establish research links with the mainland because of the present political climate, and so the United States remains the key source of scientists and research links.

Job squeeze in the West

A complicating factor is the recent tightening of the job situation for researchers in the United States. This is particularly the case in companies like IBM and Bell Laboratories that have employed many Chinese, but which in the past few years have scaled back their basic research operations, causing many to move to universities or back to Asia. However, the majority of Chinese mainland scientists still prefer to stay in the United States (see page 14).

Europe is not such a strong magnet. Nevertheless, there are thousands of Chinese scientists there, particularly at the graduate and postdoctoral level. But, unlike the United States, there are very few job opportunities beyond the postdoctoral level, and thus there is a stronger tendency for Chinese in Europe to seize opportunities to return to Asia.

Many recent young returnees to Asia await their opportunity to go back to China itself. "China welcomes top people back," says Yan Hong, who has taken up a position at the new Institute of Molecular Agrobiotechnology at NUS in Singapore after doing postdoctoral research at a company in the United States. "I hope to return when I have established a name for myself and can head a laboratory."

Cultural factors also play a role. Many

returnees have a strong mission to give something back to China. A notable example is Chen Ning Yang, Nobel prize winner for physics in 1957 for his work on weak interactions, who now holds a joint appointment at the Chinese University in Hong Kong and the State University of New York at Stony Brook. When he visited China in 1972, having left in 1945, he says that he decided he had "a responsibility to help build a bridge between the two countries ... to help China in her drive towards developing science and technology".

Returnees like Yang are building links with researchers in China as a means of fulfilling their dream of helping the motherland. As a result, there is an influx of Chinese scientists direct from the mainland to the booming economies of Asia. At NUS, for example, 60–75 per cent of the rapidly growing population of over 1,000 graduate students in science and engineering are from the mainland. The same is true of the nascent population of postdoctoral fellows.

Strong family ties are another factor bringing people back. "The fundamental unit in Chinese culture is not the individual, it is the family," says Yang. "To return to the family is not only the most desirable thing, it's the only proper thing." Fong Shih, who after 30 years in the United States is returning to Singapore to head the soon-to-be-opened Institute of Materials Research and Engineering at NUS, points out that many returnees in their late forties and fifties have



Hong Kong University of Science and Technology (HKUST) is one of the most dramatic examples of new institutions that have drawn Chinese scientists back to Asia. Opened in 1991, it has rapidly expanded its faculty members to more than 500, over 80 per cent of them of Chinese descent. The majority have come from top institutions in the United States and a few from Europe. But many of these — about a hundred — originally came from the Chinese mainland, where they did their undergraduate degrees before going to the West. There are also about 100 visiting faculty from the Chinese mainland, and about 160 of the approximately 1,000 graduate students are from the mainland, too.

parents in their seventies. "They want to come home before they go to heaven."

Shih, an ethnic Chinese from Singapore, "struggled" over his decision to return. With a PhD from Harvard, he has a tenured position at Brown University, NSF grants and PhD students. But in Singapore he can start "from scratch". "Some of the best minds in the United States are Asians. Many are now returning and next century we will compete at a very high level with the West."

David Swinbanks & Elisabeth Tacey

Collaboration with mainland China

RETURNEES who have their roots in China are particularly keen to develop research links with the mainland. One example is Leroy Chang, the dean of science at Hong Kong University of Science and Technology (HKUST). Born in Henan in 1937, Chang migrated first to Taiwan at the time of the communist takeover. After an undergraduate degree at Taiwan National University, he went to the United States, first to a small university and then to Stanford University to do a PhD. He then spent 30 years at IBM Watson (with a one-year break at MIT), where he gained fame for his research on quantum wells and superlattices. He joined HKUST in 1993.

Chang says that, although he didn't know it, he was just "following the crowd". One of his compatriots, for example, Bill Chen who also went to Taiwan National University after fleeing

the mainland, then to Stanford, and ended up at Bell Laboratories working in microelectronics for 26 years, is now in Singapore heading the Institute of Microelectronics. Both are forging links with the mainland: Chang has set up a joint life science and biotechnology laboratory with the Chinese Academy of Sciences that will open on the HKUST campus next year and further joint laboratories, for example in microelectronics, are planned, and Chen is establishing links with Chinese universities.

Chang's links go back to 1975 when he visited China with a small group of top US physicists. "That really made a difference... I was the only Chinese and after I returned to IBM, many Chinese came every year to see me," he says. "Now these people are presidents of universities and institutes. So collaborating is very easy. We have known each other for years." **D.S.**



Chang: forging links with mainland China.

Is it worth leaving the US science base?

THERE are about 120,000 scholars and students from the Chinese mainland in the United States. Most are young scientists and engineers who have arrived since 1980, following the end of the Cultural Revolution. By the early 1990s, the number of science and engineering PhDs awarded to Chinese in the United States exceeded the number awarded in China itself; they also became the single largest group of foreign students within the United States, receiving 2,751 PhDs in 1995, about 10 per cent of the total. (Taiwan was second with 1,239).

For China, the emigration of so many talented people represents a brain drain of considerable proportions. But a large and successful expatriot community also represents a huge potential resource, as China seeks to expand its own scientific and technological base. Will this potential be realized in the same way it is already being realized in the Asian 'tiger' economies?

Most Chinese arrived in the United States with the expectation of returning home after completing their studies, but the suppression of the Beijing "democracy wall" protests in 1986–87 forced many to rethink their plans. For example, Xiao-Fan Wang of

Duke University was at that time a post-doctoral fellow in Boston, and planned to return to a job at Beijing University, China's leading university. But he was also active in Chinese student circles, and after he signed a petition protesting at the clampdown, his job offer was withdrawn. Wang later learned that his name had been listed in a classified document that was circulated to high-level Chinese government officials.

Chinese politics and identity

The political repression culminated in the Tiananmen Square massacre of June 1989. In response to human rights concerns, the US government decided in 1992 to grant permanent resident status to some 80,000 Chinese citizens who were living in the United States at the time of the massacre.

The Chinese academic community in the United States thus had good reason to feel betrayed by their government. But they maintained a strong sense of Chinese identity, and since the early 1990s there has been a sea-change in their relationship with China. In part this is due to a more open attitude by the Chinese government, but there has also been something like a

"coming of age" within the expatriot community. As Xiang-Dong Fu, an assistant professor at the University of California at San Diego, puts it, "we feel a sense of responsibility, and now that we are becoming more established here, we want to do something to help China".

So far, few have returned permanently. Ray Wu of Cornell University, who left China in 1949, was involved in placing Chinese students in US graduate programmes in biochemistry throughout the 1980s; he estimates that of some 450 students who went through his programme, less than 4 per cent have gone back to take jobs in China. Wu suspects that outcome is typical, and while the exact figures are uncertain, a recent NSF survey reported that of all the Chinese students who obtained US PhDs in 1992, almost 90 per cent planned to stay in the United States. This is a sharp increase from the figure two years earlier (60 per cent), before most were granted permanent resident status.

Attitudes may change if the political situation improves in China, but many difficulties will face those who do decide to return — not least, a much lower ►

Singapore taps into Chinese talent and resources

It may seem odd to set up an institute for agricultural research in Singapore, which hardly has a square metre of arable land. But the aim of the new Institute of Molecular Agrobiolgy (IMA), affiliated with the National University of Singapore (NAS), is not to work on agriculture in the island city state. Rather, returnee Chinese scientists at the institute will work with colleagues in the Chinese mainland to develop, among other things, genetically altered pest-resistant cotton.

IMA is one of several new institutes set up by the Singapore government to develop a research and development infrastructure. Others include the Institute of Molecular and Cell Biology, which has already established its name in the world of science, and the Institute of Microelectronics, which is headed by Bill Chen, a returnee from Bell Laboratories.

IMA has recruited several young Chinese mainland scientists from Europe, where they had encountered a "glass ceiling" with no job opportunities beyond the postdoctoral level. These include Wei-Cai Yang, who in 1990 went to an agricultural university in the Netherlands from Lanzhou University (west of Xian in central China) under a Europe–China programme for postgraduate students. He turned down a postdoctoral position in Atlanta in the United States in favour of

the job in Singapore because of a desire to return to Asia, and because his wife and son are happier in the bilingual environment of Singapore, where Mandarin Chinese is widely spoken and taught.

Yang is doing basic research on plant genetics using molecular techniques. But he also has several colleagues direct from the Chinese mainland who are working on a more practical US\$25-million project to develop cotton that is resistant to bollworm pest.

China is the world's leading producer of cotton, but production has fallen drastically from 28 million bales in the mid-1980s to 18 million bales. Under a joint venture between Delta and Pine Land Company and Monsanto of the United States with Imagen Holdings, an investment arm of IMA, Monsanto technology will be used to introduce a bacterial gene into cotton that produces a toxin to kill the pest. Some adaptation of the US technique is required because Chinese cotton differs slightly from the US variety. The "Singapore–China" connection between Chinese scientists will help the US company license its technology with the Chinese government, says IMA acting director Hong-Woo Khoo.

There are several other projects on agricultural products in progress under a Singapore–China Biotechnology Pro-

gramme established between IMA and the Chinese Academy of Sciences last December. "We know the right people in China for collaboration. We plan to cultivate such collaboration. China is good at breeding plants, while we here are good at molecular biological approaches," says Yan Hong, another mainland returnee who came to IMA from the United States.

Returnee Chinese scientists at other institutes at the NUS are developing similar collaborations. For example, Bill Chen's Institute of Microelectronics is working with Fudan University in Shanghai on the characteristics of sheets and films for packing technology for microelectronic products. And the university also has a "big project" with Qinghua University in Beijing to develop the next generation of fuzzy logic systems for control of large industrial plants, says deputy vice-chancellor Chang-Chieh Hang.

"The unique thing about China is that Chinese professors are involved in the building of industrial plants. They bring new ideas and facilities to factories. Professors have a lot of access to pilot plants where we can test out things on a large scale," says Hang. Furthermore, China has plenty of talented basic researchers who can fill the lack of such manpower in Singapore.

David Swinbanks

► standard of living. Moreover, many have already made substantial scientific contributions, and would find it difficult to maintain international competitiveness given the shortage of funds and limited research infrastructure in China.

Nevertheless, the balance is shifting. Jobs and funds are becoming tighter in the United States, while at the same time reform is under way in the Chinese academic system. Because of the generation gap created by the Cultural Revolution, there are too few middle-aged academics to replace those approaching retirement. It is therefore possible to rise rapidly through the system.

Zhang-Liang Chen, for example, obtained a PhD from Washington University in 1987, and at 35 is already a vice-president of Beijing University. (Chen received widespread publicity for his claim to have cloned dinosaur DNA, despite scepticism among many experts.) And the Chinese Academy of Sciences aims to recruit 100 people, most of whom will be foreign-trained, to leadership roles in its many institutions around the country.

One of those recent recruits is Gang Pei, who returned last year from Duke University to a position at the Shanghai Institute for Cell Biology, funded in part by the German Max Planck Society. While not denying the difficulties of doing research in China, Pei notes that people who returned to Taiwan or South Korea a few decades ago have in many cases risen to top leadership positions as their countries prospered. "It's like buying stock," he says. "The aim is to buy low and sell high, and the profit is always proportional to the risk. Look at Japan; they are talking about a 50 per cent increase in their research budget. That could be China in the future."

Alternatives to resettlement

Many who are not yet willing to risk their careers by returning permanently are nevertheless looking for other ways to participate. One way is to host visiting scholars from China, who come for short visits and take back expertise with them. Another is to collaborate with colleagues in China, and there are various organizations that sponsor such exchanges.

Eric Shi, for example, a cancer researcher at Long Island Jewish Medical Center, was enabled by the China-Cornell Fellowship Program to establish a collaboration with Tianjin Cancer Institute and Cancer Hospital; in one of the largest projects of its kind anywhere in the world, they are using the hospital's collection of biopsy material to look for molecular markers of breast cancer progression. (Breast cancer is rising sharply in China, as in many other Asian countries; this is due in part to the falling number of

pregnancies, making it a serious concern for a government committed to a one-child-per-family policy). In addition to such exchange programmes, many people maintain informal contact with their former institutions, bringing equipment, reagents and information when they return for family visits.

In one innovative plan, three molecular

The Tiananmen Square massacre of June 1989 was a turning point for many Chinese within the United States.

neurobiologists based in the United States are setting up a lab in the Shanghai Life Science Research Centre, which they will run jointly from their home institutions. Bai Lu of NIH, Lin Mei of University of Virginia, and Yi Rao of Washington University plan to work closely with Linyin Feng in Shanghai, communicating by e-mail and visiting Shanghai in rotation several times each year; meanwhile, students and post-docs from Shanghai will make reciprocal visits to the United States. Says Lu, "our goals are to establish a modern neurobiology lab that will be recognized by the world, and to train a group of young scientists who will play an important role in China's scientific future".

Several organizations have been formed in the past few years to facilitate scientific collaborations between China and the United States. The Chinese Biopharmaceutical Association (CBA) provides contacts and expert advice to the nascent Chinese biotechnology industry, while the Sino-American Pharmaceutical Association (SAPA) does the same for the pharmaceutical industry, providing advice not only on technology but also on marketing and regulatory issues. According to Guo-Liang Yu of Human Genome Sciences Inc., who is president of CBA, their members are mainly Chinese people working in US universities and industries, who volunteer their time to help companies in China develop their products and markets.

And it is not just a one-way street, says Junyan Hong of Rutgers University, former chairman of SAPA: there are also potential benefits from traditional Chinese medicines, most of which have not yet been subjected to rigorous testing by western standards. One that has is artemether, a traditional herbal cure dating back to the Sung dynasty.

Artemether was recently shown to be as effective as quinine in preventing death from malaria. Hong hopes that by helping researchers in China to design proper trials, it may be possible to identify other new products that can be sold worldwide.

The increased contact with the West over the past two decades is now starting to influence official attitudes in China. That much is clear, for example, from the experience of Chia-Wei Woo, president of Hong Kong University of Science and Technology, who was born in Shanghai and who, when in the United States as chairman of the department of physics and astronomy at Northwestern University, was a key participant and organizer of visiting scholars programmes with China.

"Those people [who participated] are now university presidents and mayors, many with backgrounds in science and engineering", he says. "This is something I keep telling people in the States. There has been a tremendous change in China. You don't need to deal with revolutionaries anymore."

China rethinks

After years of disapproval, the Chinese government is now coming to terms with its "scientific diaspora". In part this is an acknowledgement of the inevitable: since so many Chinese scientists now have permanent resident status in the United States, China cannot compel them to return. In any case, it could not possibly absorb them all into its academic system or fund them at a level where they could remain productive. China itself cannot invest heavily in basic research, and its emphasis for the immediate future must necessarily be on practical applications. Nevertheless, it recognizes that to maintain its technology base over the long term, it must remain in contact with the world scientific community, and the best way to do so is to cultivate a good relationship with Chinese scientists abroad.

Xiao-Fan Wang, the former dissident once denied a job for his activities, was among 20 young faculty members who were invited to the Chinese Embassy in Washington DC earlier this year. "They told us that they were pleased to see us here in the United States, and that they hoped we would stay and continue to be successful," he says. And the government is willing to listen; SAPA members recently met with a ministerial delegation to discuss strategic planning and regulatory standards for the Chinese pharmaceutical industry, and many of their recommendations are now being implemented. "Now, our members can stand up and contradict high-level government officials in public", says Junyang Hong. "Ten years ago, that would have been unthinkable."

Charles Jennings

Chia Hires/Frank Spooner Pictures

Application for patent opposed

SIR — As Gluckman *et al.* recently reported (*Nature* 382, 108; 1996), European and US research groups are protesting against the application by the US company Biocyte Corporation for a patent, already granted in Europe, on the use of stored haematopoietic cells from umbilical cord blood.

Their complaint, amplified in a News story by Declan Butler (*Nature* 382, 99; 1996), is based on the belief that the patent is unethical: if these patents are enforced, the use of cord-blood cells for transplantation will not conform to the resolution of the International Society of Transplantation establishing the basic principle that no part of the human body should be commercialized and that donation of organs or cells should be free and anonymous.

Since the patent application was opened to the public in Japan in January 1996, two companies have challenged it. A number of clinicians, scientists and volunteer groups for cord-blood banking will present a written opinion to the Patent Office at the Ministry of International Trade and Industry of Japan opposing the patent. In Japan, there had been almost 800 bone-marrow transplantations from non-relatives through the Japan Marrow Donor Program up to June 1996. Nevertheless, many patients are still waiting to find an HLA-matched donor. At least ten cord-blood transplantations from siblings have been carried out in Japan in the past two years. And there is now a movement to establish a cord-blood bank.

The Bone Marrow Transplantation Research Group organized by the Japanese Ministry of Health and Welfare continues to study the clinical value of cord-blood transplantation. All clinicians and scientists are invited to join this group, which will develop standard protocols, including collection, separation and cryopreservation, as well as standards for clinical applications for safe, successful and ethical transplantation.

Such a movement should not be hindered. We therefore join Gluckman *et al.* in protesting from an ethical point of view against the granting of this patent to Biocyte Corporation. We are also concerned that this patent may discourage and threaten the activities of non-profit cord-blood banks, clinicians, patients, parents and the volunteers who support them.

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Sham review

SIR — Your News item "Report advises against privatization" (*Nature* 382, 569; 1996) highlights the secrecy in which the current 'prior options review' of Natural Environment Research Council research institutes is being held. It is not good enough for the Department of Trade and Industry spokeswoman to say that "[r]esearch councils do know what the recommendations are" if they are not allowed to tell us, or even to inform their own institutes. It is not only scientists who have been critical of the process. The chairman of the House of Commons Select Committee on Science and Technology, which has a Conservative majority, has expressed disapproval and written that "we are forced to comment on the basis of hypothesis and rumour" (HC 643 of 1995–96).

One may be forgiven for thinking that the prior options exercise is a sham and that the secrecy in which it is being conducted is to enable ministers to do what they wish (privatize, close down?) even if their experts have recommended otherwise.

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By any other name...

SIR — Sir John Houghton's defence of the revision of Chapter 8 of the recent Intergovernmental Panel on Climate Change (IPCC) report (*Nature* 382, 665; 1996) says at several points that what was done was done in accord with "IPCC procedures". Most will accept that, but is not the important question whether the procedures match the task?

Take much-mentioned 'peer review'. Journals use it to help them decide what will interest their readers, grant-making agencies to decide how best to spend their funds, but IPCC believes that peer review will guide it (and the rest of us) to a consensus on climate change. What if consensus is unattainable?

I share IPCC's suspicion that global warming may be the most serious threat in sight to our civility, but I also believe that IPCC's 'procedures' may do as much harm as good. Most of us now know that greenhouse gases are potentially a problem and also that there are persisting uncertainties about the immediacy of the risk.

What we need is not a periodic restatement of the current approximation to a consensus view addressed to "policy-

makers" (civil servants?), but a periodic assessment of the continuing uncertainties in the climate models and a continuing review of the 'peer-reviewed' literature, dissident and otherwise, that carries weight in the research community. That way, in due course, IPCC's authority would not require consensus.

As things are, IPCC should recognize that, however robustly it resists "those with various political agendas", consensus-building is inherently a political process whose practitioners are called "spin-doctors" in other trades.

John Maddox

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Pseuds' corner

SIR — Your Daedalus column is, as always, fascinating, but "The highest spin" (*Nature* 382, 212; 1996) must have caused some other jaws to drop besides mine. As the basis for his newest cleverness, David Jones describes how "rotating winged seeds, such as those of the sycamore tree, have spun sedately to the ground each autumn". The startled reaction occurred because the sycamores that I am familiar with have spherically symmetrical fruit heads (fruit balls typically a little over an inch in diameter) which often persist throughout the winter on the tree and, if they fall, behave, if anything, more like Newton's apple than a helicopter. In the United States and Canada, we see seeds of maple trees (*Acer* sp.) behaving in the fashion ascribed in the Daedalus column to sycamores.

After some puzzlement about the apparent aberrant reproductive behaviour of English sycamores, I concluded that this is — again — an international misunderstanding based on nomenclature differences, and that Jones is referring to a type of maple (*Acer pseudoplatanus*) while what we in North America call a sycamore is a type of plane tree (*Platanus occidentalis*).

Incidentally, it turns out that there is yet another type of tree called a sycamore, the sycamore tree mentioned in the Bible, which is a fig tree (*Ficus sycomorus*) of the Near East, which was notable for its use in ancient Egypt for making mummy cases.

Accordingly, may I suggest that, instead of designating his newest invention a "sycacopter", Jones considers renaming it an "acercopter".

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Storing diffraction data

SIR — *Nature* must consider structural biology to be of some interest to its readership, as almost every issue contains a new macromolecular structure. We therefore regret your publication of the letter from Hooft *et al.* on errors in protein structures¹. The nonspecialist reader will immediately draw the conclusion that the crystallographic structures in the Protein Data Bank (PDB) are extremely unreliable. The quality of structures deposited at the PDB is indeed variable, but since the spate of high-profile errors that led to the Commentary by Brändén and Jones², the community has tried hard to develop new methods and protocols to make such errors less likely to occur or to escape attention.

We would like to make the following comments:

- *Nature's* Correspondence section is not a suitable place to publish the results of a validation study of the PDB.

- Many listed items are simply not errors. From the wealth of structural knowledge in the PDB, we now know a lot about macromolecular preferences. For example, although proteins show clear preferences for certain main-chain conformations, an outlier in a Ramachandran plot is simply an outlier, it is not necessarily an error! In one enzyme family, the α/β hydrolases, the

active-site nucleophile always has a so-called 'disallowed' conformation. Indeed, we would expect up to 5 per cent of all non-glycine residues to fall in 'disallowed' regions of this plot (G. J. K. & T. A. J., to be published).

- Stereochemical knowledge has been used in the refinement of most structures in the PDB, because the macromolecular crystallographer is usually limited by the resolution of the diffraction data. Improvements have been made in the dictionaries describing this information and in the refinement protocols. Using these newer dictionaries on older coordinate sets, refined with older protocols, is sure to produce many outliers. Even for models refined with the most modern dictionaries, stereochemical outliers can only indicate potential errors. On the other hand, it is perfectly possible to make a structure that has excellent stereochemistry and is totally wrong³. Good stereochemistry is not proof of a correct structure, although crystallographers usually provide these statistics as if it were.

- One cannot always use the coordinates without reading the remarks contained in the PDB entry or original publication. This is difficult for a computer program to do. For example, one structure is flagged as

having no fewer than twelve D-amino acids that are, therefore, counted as errors. Even a cursory glance at the PDB file, however, reveals that this is the structure of gramicidin (an antibiotic made up of both L- and D-amino acids), which is one of the highest-resolution structures in the database. Other cases of D-amino acids may well be examples of poor stereochemistry. In another example, the entry 1GRH has been flagged as having a particularly large specific volume, V_m ($954 \text{ \AA}^3 \text{ Da}^{-1}$, whereas one would expect something in the range 2 to 3), because the authors have deposited the coordinates of a single modified residue, as described in the file.

- Insufficient care has been taken in preparing the report. Some of the very close contacts that are flagged as errors arise because atoms sit on special positions in the unit cell. It is not uncommon for a water molecule or ion to sit on a crystallographic rotation axis so that the distance to one or more crystallographically related copies of itself will be zero. This is not the major reason for this class of error (the second most common class according to the authors), but we mention it to highlight the care that must be taken when preparing a report of alleged errors. Like crystallographers, Hooft *et al.* are human and their software has some shortcomings. For example, in the report on 2DRI, a ribose-binding protein from *Escherichia coli*

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which has been refined to a resolution of 1.7 Å (ref. 4), most of the atoms itemized as lacking hydrogen bonds actually form hydrogen bonds to the bound ribose, while others involve residues that are external.

• Many book-keeping errors have been located. For example, some of the large V_m values are due to errors on the PDB CRYST1 card where the crystallographer has specified the number of noncrystallographic symmetry partners instead of the number of molecules in the cell. In other files, the temperature factors and occupancies may be in the wrong order. These are not errors in the structures but errors in the PDB files.

• Now that *Nature* requires the deposition of coordinates, the next step should be taken and the deposition of diffraction data should also be made mandatory, if necessary with an appropriate hold period. Many of the alleged errors listed by Hooft *et al.* could be properly evaluated if the experimental structure factor data were available. In many instances, these data may be lost to the community because of the rapidly changing world of computers where the optimal method of back-up is

not constant. Deposition of diffraction data is probably the best way to improve the quality of the structural database and to ensure long-term archiving, but the process must be enforced. For example, a couple of months ago, two more-or-less identical structures were published in *Nature* and *Science*. The *Nature* coordinates have now been released, but the *Science* ones are still nowhere to be found (and we are still waiting for a response from the authors about their availability). Without the experimental data, one can suspect that there are problems in a particular structure but, except for trivial chirality errors that no-one would argue with, there is no proof. We have our own prejudices about how structures should be refined, and in particular we believe that low-resolution structures are being over-refined³. We have indicated some examples of this even at moderate resolution⁵, but without the diffraction data there is little more that can be done.

• It would have been interesting to see how the 'errors' depend on the year of deposition. We think the quality of the deposited structures is improving, although the methodology being used is by no means uniform. The free R -value (R_{free}) has recently been introduced⁶ as an aid in recognizing problems during crystallographic refinement. It can also help the crystallographer in designing the optimal refinement

protocol. We have monitored its use in structures published between January and June 1996 (ref. 7). This shows a remarkable variation that depends on the journal in which the structure is published. In three journals (*Nature*, *Cell* and *Structure*), more than 80 per cent of the structures have R_{free} quoted. In *Nature Structural Biology* and in *Science*, about 65 per cent do so, while in *Acta Crystallographica D*, *Journal of Molecular Biology*, *Proteins*, *Protein Science* and *Biochemistry*, fewer than a third of the structures have a quoted R_{free} .

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Fate of conference abstracts

SIR — Given the number of scientific conferences held worldwide, and the amount of time physicians and scientists spend attending them, we decided to investigate the fate of abstracts presented at these meetings. We wanted to know how many ultimately became papers published in peer-reviewed journals, and, if published, how closely they resembled the original abstract.

We randomly selected 100 abstracts presented at the 1992 annual scientific meetings of each of four associations: the American Heart Association, the Feder-

percentage, if not a majority, of abstracts presented at scientific conferences do not evolve into papers. However, abstracts in general present data in significant advance of any subsequent publication in peer-reviewed journals.

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this so-called radium was really barium — the crucial result that represented the discovery of fission. Thus, “good solid chemistry got things on the right track”², but Strassmann, who designed the work and carried it out, remained “in the shadow of the sensation” as his biography is entitled³.

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PUBLICATION RATES FOR ABSTRACTS

	<i>Circulation</i>	<i>FASEB J.</i>	<i>Gastroenterology</i>	<i>Neurology</i>	Total
% Published as peer-reviewed papers	30.0 (30)	28.0 (28)	42.0 (42)	41.0 (41)	35 (141)
% Not published	70.0 (70)	72.0 (72)	58.0 (58)	59.0 (59)	65 (259)
Of the abstracts published in peer-reviewed journals:					
Same first and last author	15	23	34	22	94
Reverse order of first and last authors	0	0	1	1	2
% Change of authors	50.0	17.9	19.0	46.3	33.3
% Change in conclusions	0	3.6 (1)	0	0	0.71 (1)
Years to publication	1.9±0.8*	1.6±0.9	2.2±0.9	1.9±0.9	1.9±0.9

Numbers in parentheses show absolute number of cases.

*Represents standard deviation.

ation of American Societies for Experimental Biology (FASEB), the American Gastroenterological Association and the American Academy of Neurology (published in *Circulation*, *FASEB Journal*, *Gastroenterology* and *Neurology*, respectively). We then searched Medline.

Of the 400 abstracts examined, 35% developed into journal articles. Publication rates varied significantly from field to field. Twenty-eight per cent of the abstracts in *FASEB Journal* were published, the lowest rate of the four journals, while *Gastroenterology* abstracts had a high of 42%. But the time to publication for the abstracts from these two journals was inversely proportional to the amount published. *FASEB Journal* abstracts were published, on average, 1.6 ± 0.9 years after the conference, while *Gastroenterology* abstracts were not published for 2.2 ± 0.9 years. Although conclusions almost never changed, either first or last authors changed one-third of the time. Again, this varied among fields. *FASEB Journal* abstract authors had the lowest turnover rates (17.9%), while *Circulation* authors changed 50% of the time.

We therefore suggest that a significant

Strassmann in the shadow

SIR — In the seven years during which the Nobel committees were thinking about the Nobel prize for the discovery of nuclear fission, a variety of solutions were considered¹.

Should the prize be awarded in chemistry or physics, to Hahn, and/or Lise Meitner, and the young Otto Robert Frisch? It is astounding that, according to the account by Crawford *et al.*¹, the other youngster involved, Fritz Strassmann, was not even considered, even though he played a key role in the discovery. It was his hypothesis that a new “transuranium element” reported in October 1938 by Irène Curie and Paul Savitch could be a mixture of the known elements radium and actinium that led to the crucial chemical experiments.

It was Strassmann who then designed an elegant chemical method which appeared to reveal the somewhat surprising presence of radium in neutron-irradiated uranium, and it was Strassmann who doubted that

1. Crawford, E., Sime, R. L. & Walker, M. *Nature* **382**, 393–395 (1996).
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Exodus enigma

SIR — Bruins and van der Plicht present evidence¹ dating the destruction of the Bronze Age settlement of Jericho at $3,111 \pm 8$ years BP, and the Minoan Santorini eruption some 45 years earlier. Assuming that the dust cloud from the Santorini eruption caused the “thick darkness” mentioned in Exodus 10:21, and the sack of Jericho is that described in the Book of Joshua, they suggest that this coincidence of dates supports the Exodus account of the Children of Israel wandering in the Sinai desert for 40 years.

But evidence (and lack of it) from other quarters casts doubt on the reliability of the Exodus account. First, no ancient Egyptian chronicles describe events resembling the Exodus, making it doubtful whether any of the Israelites' forebears were ever slaves in Egypt. Second, the “Mountain of Sinai” described in Exodus 19:16–20 is recognizably an erupting volcano: the eruption appears to be of the vulcanian type, associated with cinder-cone volcanoes. There are no volcanoes in Sinai. In the Middle East, volcanoes that have been active during the past five thousand years are found in Syria, Saudi Arabia, Yemen and South Yemen². They are all of the ‘volcanic field’ type, except for Jabal Hamman Demt in Yemen, which is a cone.

It therefore seems that the Book of Exodus contains an amalgam of folk-histories of more than one nomadic tribe originally inhabiting widely separated regions in the Middle East.

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Earthquakes: slow down for safety

Paul Segall

In December 1992, part of the San Andreas fault ruptured. No damage was caused, because instead of lasting for a few seconds, this earthquake sequence took a week. So why are other earthquakes fast?

Is the term 'slow earthquake' a contradiction? It might seem so, as earthquakes typically propagate at velocities limited only by the speed of sound in rock — several kilometres per second. As the rupture propagates, the fault surfaces slip by up to several metres (depending on the size of the quake) in the course of one or two seconds, causing seismic waves to be radiated into the Earth. The more slowly the fault slips, the less efficiently it generates seismic waves; if fault slip is slow enough, no waves are radiated and the earthquake is silent. Indeed, if all earthquakes were slow they would not be very hazardous, because ground shaking causes nearly all the damage in earthquakes. Nevertheless, slow fault slip, or creep, does occur, notably on the central San Andreas fault in California. Most creep events, however, are small and shallow. Because they generate little, if any, seismic energy, slow earthquakes are best studied using highly sensitive strain meters. Earthquakes lasting as long as a few hours have been detected in the past. Now, on page 65 of this issue¹, Linde *et al.* report a week-long sequence of slow earthquakes, which would have been equivalent to a magnitude 4.8 earthquake had it occurred rapidly.

Why do faults sometimes slip slowly and harmlessly, and at other times catastrophically? To answer this question, it is useful to consider the simple spring-slider earthquake model described by G. K. Gilbert in 1884. It was Gilbert who first explained how earthquakes were caused by the accumulation of strain in the Earth's crust: "... this strain increases until it is sufficient to overcome the starting friction on the fractured surface. Suddenly, and almost instantaneously, there is an amount of motion sufficient to relieve the strain, and this is followed by a long period of quiet, during which the strain is gradually reimposed"².

Gilbert, who was decades ahead of his time in understanding the mechanics of earthquakes, devised the following analogy²:

"Attach a rope to a heavy box and drag it slowly, by means of a windlass, across a floor. As the crank is turned, the tension in the rope gradually increases until it suffices to overcome the starting friction, as it is called.

"Once started, the box moves easily, because sliding friction is less than starting

the motion of the Earth's tectonic plates; an elastic element (the rope), which represents the Earth's crust adjacent to the fault and its ability to store elastic energy; and the frictional fault surface (the box-floor interface).

We now know that Gilbert's concept of friction was too simple; the transition from the 'starting' or static friction to sliding friction is not instantaneous, but occurs gradually with increasing slip displacement³. In fact, if the transition were instantaneous, slow earthquakes, such as those found by Linde *et al.*, could not exist. Modern laboratory experiments show that the coefficient of friction depends on slip speed and past slip history. If sliding continues at a constant rate, the friction coefficient approaches a unique steady-state value⁴.

Whether or not the slider exhibits jerky, stick-slip motion or continuous, stable sliding depends on the interaction of the frictional properties and the elastic loading system. Unstable, accelerating slip occurs only when the frictional strength decreases more rapidly than the elastic element unloads. So stiff systems favour stable slip whereas compliant systems favour unstable stick-slip — try replacing Gilbert's rope with a steel cable or a bungee cord. Stability also depends on the normal stress acting on the fault surface (that is, the weight of Gilbert's box). High normal stress (a heavy box) favours unstable, stick-slip motion, whereas low normal stress favours stable sliding. Of course, the frictional properties enter into the stability equation. Slip can be linearly unstable when the steady-state friction decreases as a function of slip speed, but is generally stable when it increases⁵.

Faults in the Earth's crust contain fluid-saturated 'fault gouge', finely fragmented rock material whose properties can greatly influence fault stability. If the fluids within the gouge are maintained at high pressure, the effective normal stress acting across the fault is diminished, promoting stable slip. These arguments point to two competing hypotheses for the stable creeping section of the San An-

IMAGE
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REASONS

Danger area: the San Andreas fault crosses the Carrizo plain, about 450 km south of San Francisco. But some sections of the fault can release their strain slowly and safely.

friction. The rope shortens or sags until its tension is only sufficient for the sliding friction, and it would continue in that state but that the box, having acquired momentum, is carried a little too far. This slacks the rope more, and the box stops, to be started only when the tension again equals the starting friction. In this way the box receives an uneven, jerky motion. Something of this sort happens with the mountain."

Gilbert's earthquake analogy includes the three key ingredients of any earthquake machine: a driving mechanism (the windlass), which we now understand to be

David Parker/Science Photo Library

dreas: that low-permeability rocks next to the fault trap pore fluids at high pressure, or that these same rocks have intrinsically stable frictional properties.

The dynamics of fluid-saturated fault zones have other complexities. As they shear, granular materials dilate, that is, they increase pore volume, because the grains ride over one another. If exchange of fault-zone pore fluids with the surrounding rocks is slow, then dilation transiently decreases pore-fluid pressure and is thus stabilizing⁵. On the other hand, fault slip generates frictional heat, which, depending on fluid transport properties, can lead to thermal pressurization of pore fluids and so to instability⁶.

Other non-fluid effects may also influence fault stability. High-frequency seismic waves trapped in the fault zone can briefly reduce fault strength, promoting instability⁷. And it has recently been suggested that differences in elastic properties on either side of the fault may lead to unstable slip pulses, ripple-like disturbances accompanied by transient decreases in normal stress⁸.

These processes can all be studied by numerical simulation, and some have been verified in laboratory experiments. Their relationship to unstable slip on natural faults, however, remains clouded because of our poor knowledge of conditions and material properties at depths at which earthquakes nucleate (typically 10 to 15 km on the San Andreas). The slow earthquakes recorded by Linde *et al.* were near the transition between the creeping segment of the San Andreas and the northern, locked segment, which in some respects may be analogous to the transition from stick-slip to stable sliding that occurs at a depth of about 15 km elsewhere on the fault. Only by comparing theoretical predictions with field and laboratory observations will it be possible to isolate those effects central to earthquake instabilities. In this way, observations of slow and silent earthquakes may lead to a better understanding of how their faster, more damaging cousins begin. □

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A growing coactivator network

Ralf Janknecht and Tony Hunter

STEROIDS, thyroids, retinoids and vitamin D all bind to nuclear hormone receptors, which interact with promoter elements to regulate gene transcription. Three reports¹⁻³, including one on page 99 of this issue², show that nuclear hormone receptors depend on the coactivator CREB-binding protein, CBP, or the related P300 protein, to trigger RNA polymerase II and thus transcription. CBP and P300 are huge nuclear molecules, consisting of more than 2,400 amino acids, and can interact with a variety of different DNA-binding factors and also with components of the basal transcription machinery⁴:

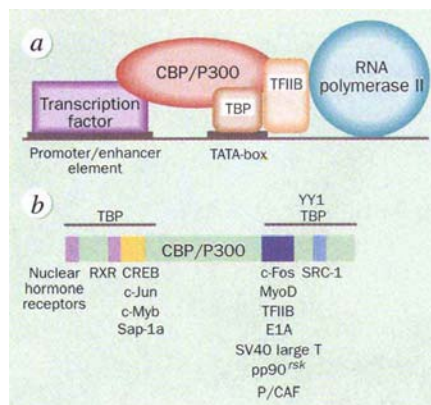


FIG. 1 a, Model of CBP/P300 bridging between a sequence-specific transcription factor and components of the basal transcription machinery. TBP, TATA-box-binding protein; TFIIB, transcription factor IIB. b, Binding sites of the various partners that interact with CBP/P300. RXR, retinoid X receptor. CREB, c-Jun, c-Myb, Sap-1a, c-Fos, MyoD and YY1 are all DNA-binding factors. E1A and SV40 large T, viral proteins that suppress activation of transcription. pp90^{rsk} and P/CAF, enzymes recruited by CBP/P300. SRC-1, coactivator which interacts with both CBP/P300 and nuclear hormone receptors.

CBP/P300 thereby links these two classes of proteins (Fig. 1). Indeed, the ability of adenoviral E1A oncoprotein and SV40 large T antigen to interfere with these interactions explains how they suppress transcriptional activation⁵.

The new reports¹⁻³ show that nuclear hormone receptors interact directly with CBP/P300 through their ligand-binding domains, and that hormone binding greatly stimulates this interaction. Two receptor-interaction domains have been mapped within CBP: the amino-terminal 101 amino acids, which bind to all tested nuclear receptors, and amino acids 356-495, which bind weakly to the retinoid X receptor but not to the retinoic acid or thyroid hormone receptors^{1,2}. Nuclear-receptor-mediated transactivation is enhanced by CBP/P300 in a

hormone-dependent manner *in vivo* and *in vitro*, and is blocked by injection of anti-CBP antibodies into cells. So the interaction between CBP/P300 and nuclear hormone receptors is essential for their transactivation function^{1,2}.

In addition to the coactivators CBP/P300, nuclear hormone receptors interact with a variety of other coactivating or silencing mediators. One of them (SRC-1) interacts with a carboxy-terminal region of CBP/P300 (refs 1, 6). Nuclear hormone receptors can bind simultaneously to CBP/P300 and SRC-1, in principle forming a ternary complex bound to DNA. This suggests that multiple coactivators may jointly modulate transactivation mediated by a single DNA-binding transcription factor. A corollary of this may be that multiple components of the basal transcription machinery are contacted, but how this finally triggers initiation of transcription remains a mystery.

A further element to the story has come from the discovery of a P300/CBP-associated factor (P/CAF)⁷, which competes with viral E1A protein for binding to CBP/P300: this competition is responsible for the counteraction of E1A-induced mitogenicity by P/CAF. Functionally, P/CAF is an enzyme that, *in vitro*, acetylates histones H4 and H3 but not histones H2A or H2B (ref. 7), and may therefore destabilize nucleosomes and promote transcription. Another enzyme recruited by CBP/P300 is the protein kinase pp90^{rsk} (ref. 8), which after Ras-induced activation and nuclear translocation can bind to the same region as E1A and thereby suppress CREB-mediated transcription. In contrast, association of pp90^{rsk} with CBP is required for induction of Ras-responsive genes, possibly because pp90^{rsk} phosphorylation of DNA-bound factors, components of the basal transcription machinery or CBP/P300 itself can enhance transcription of such genes.

Loss of one CBP allele apparently causes Rubinstein-Taybi syndrome, which is an autosomal dominant inheritable disease associated with mental retardation, broad big toes and thumbs, and facial abnormalities⁹. This suggests that CBP can be a limiting component within the cell, and that competition for CBP may allow cross-talk between different signalling pathways. Consistent with this, nuclear hormone receptors can antagonize the AP-1 transcription factor, which is composed of Fos and Jun proteins, and overexpression of CBP or P300 abrogates the inhibition of AP-1 function by hormone-bound nuclear receptors. In addition, a synthetic ligand abolishes interaction of the retinoic acid receptor with CBP/P300,

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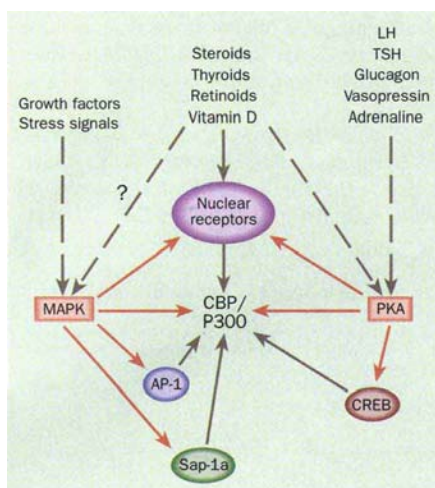


FIG. 2 Integration of signalling pathways resulting in the activation of gene transcription by CBP/P300. LH, luteinizing hormone; TSH, thyroid-stimulating hormone; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; AP-1 and Sap-1a, DNA-binding transcription factors which are activated by MAPK; CREB, regulatory protein, so called because it binds to the cyclic-AMP-response element (CRE) found in the regulatory region of genes activated by cAMP. CBP was first identified through its interactions with CREB — hence its abbreviation from CREB-binding protein.

and consequently suppression of AP-1 transactivation is no longer observed¹.

Conversely, the function of nuclear hormone receptors can also be impaired by sequestration of CBP/P300. For instance, upon phosphorylation of the cyclic-AMP-dependent protein kinase A (PKA), CREB acquires the ability to interact with CBP/P300 (ref. 4) and phosphorylated CREB suppresses retinoic-acid-induced gene activation¹. Also, different nuclear hormone receptors can antagonize one another in a ligand-dependent fashion¹⁰. So different transcription factors activated by diverse signalling pathways may interfere with

one another by competing for limiting common cofactors (CBP and P300), and this regulatory squelching may — more often than anticipated — contribute to the control of gene activity¹¹.

In many cases, steroid hormones induce the phosphorylation of their cognate receptors at sites that are potentially recognized by mitogen-activated protein kinase (MAPK) or PKA. However, the physiological role of nuclear-receptor phosphorylation is ambiguous, although it may sometimes enhance transactivation potential^{12,13}. Furthermore, protein kinase activators such as epidermal growth factor or cyclic AMP can induce transcription mediated by nuclear receptors, either autonomously or synergistically with hormone^{14,15}. This may occur through direct phosphorylation of the receptors, but could also be due to the phosphorylation of the coactivator CBP by MAPK or PKA (Fig. 2), which has been shown to enhance the coactivation potential of CBP⁴.

ANTIBODY CATALYSIS

The key is in the pocket

Stephen J. Benkovic

FOR the past 10 or more years, antibodies have been considered the most promising candidates for mimicking enzyme catalysis. But are their catalytic properties due to complicated specific binding interactions, or to something more basic? The report by Hollfelder *et al.* on page 60 of this issue¹ describes catalysis of a simple elimination reaction by a nondescript albumin, known not for its enzymatic properties but for its role in ion transport. The rate acceleration achieved is remarkably similar to that observed for a catalytic antibody promoting the same reaction. Their report rekindles questions about how best to discover systems capable of enzyme-like catalysis.

There is a rich historical record of attempts to imitate the properties of enzymes, specifically their nearly absolute specificity and their ability to accelerate reaction rates by more than a million-fold². The first systems were intramolecular, mimicking the chemical transformations that occur within the enzyme-substrate complex. In particular, amide and ester bonds were broken by a variety of reactive functions acting as nucleophiles or general acids/bases.

There are two effects that contribute to the rate acceleration in these experiments: the translational motions of the reactants are reduced, increasing the probability of an encounter, and their molecular geometry is altered to facilitate the chemical transformation. In some cases in the experiments of ref. 2 where the reacting centres were constrained at an appropriate

distance and orientation, rate accelerations approached 10^8 . But because the 'catalyst' and substrate were covalently bound, this was not true catalysis — there was no turnover of product. Enzymes, meanwhile, have evolved the ability to bind their substrates, carry out chemistry, and release their products in order to start another catalytic cycle. The first artificial systems to have that property were micelles that incorporated the substrate and acted to increase or decrease the reaction rate depending on electrostatic considerations. Others were simple polymers like polyvinylimidazole, which showed the Michaelis-Menten kinetics associated with catalysts that bind their substrates with high specificity. Even though most of these systems had no specifically crafted binding sites, unlike the cyclodextrins and macrocycles of later vintage, the rate accelerations were generally modest (10^2 – 10^4 over background).

One of the fundamental ideas for explaining enzyme catalysis is that the active site of the enzyme selectively lowers the free energy of the substrate in its transition-state geometry³. The hapten (the molecule used to induce the immunological response) in the case of catalytic antibodies is a stable molecule whose dimensions and electronic distribution approximate those of the reactant's transition state. So one might have anticipated that the rate accelerations observed with catalytic antibodies would be greater than the values of 10^2 – 10^4 generally observed.

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(These rate enhancements are given by the ratio of k_{cat} , the first-order rate constant for chemical transformation of the antibody-substrate complex, to either the first-order rate constant for the uncatalysed reaction or the second-order rate constant in the case of a bimolecular reaction.)

Some of the blame for not attaining the high kinetic efficiency observed with enzymes surely lies in our inability to synthesize accurate transition-state analogues. But another factor is the need of the immunological response to achieve an antigen-antibody union on the surface of the protein, rather than tucked away from the solvent in a protein pocket as with substrate-enzyme interactions. The antibody might not generously provide the amino-acid side-chain needed for the chemical transformation, nor position it with the required sub-ångström precision.

So why are enzymes special? Perhaps there has been too great a focus on a comfortable explanation couched in terms of reactant transition-state stabilization. What counts is the difference in free energy between reactants and the transition state, and how this gap is reduced by the catalyst. It is doubtful that the enzyme's affinity for the transition state is actually billions of orders of magnitude greater than its binding of the substrate, because the difference between the two is often only a subtle change in dipolar forces. The hidden flaw in such estimates may be that they don't account for the importance of the enzyme's ability to organize its substrates and environment, before the transition state is achieved, into a reactive configuration and provide a stable environment for the reaction. This organization of the reaction environment provides a template for the chemical transformation by optimally locating functional groups and orienting fixed water molecules.

In contrast, the same reaction in solution may well require considerable solvent reorganization in order to proceed, and the neglect of that reorganization appears as 'transition-state binding'. Molecules with 'adventitious pockets', possessing solvent microenvironments and constellations of acid/base groups, will be found occasionally that have catalytic properties similar to those of molecules with more sophisticated programmed construction. This will be more probable for reactions that proceed quickly without catalysis, and are not mechanistically complex, such as the elimination reaction studied by Hollfelder *et al.*, which is sensitive to solvent reorganization and the strength of the catalytic base.

The enzyme's ability to bind reactants in a manner that preorganizes the enzyme-substrate complex for high reactivity, and the subsequent release of products, reflects an interplay between structural elements of

the protein as the process progresses. Often, the substrate in the reactive complex is totally hidden within the active-site cleft, enclosed by structural motifs that fold over the substrate after binding. At present it will be more by serendipity than design if the majority of all such elements appear in any artificial system.

On the other hand, as exemplified by the report from Hollfelder *et al.*, the clever exploitation of pre-existing structural motifs — especially off-the-shelf proteins with adventitious pockets — may lead to systems with enzyme-like properties. Given the difficulty of the

challenge, it is no wonder that random mutagenesis or combinatorial methods have proved so popular. □

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NEUROBIOLOGY

Awakening the brain

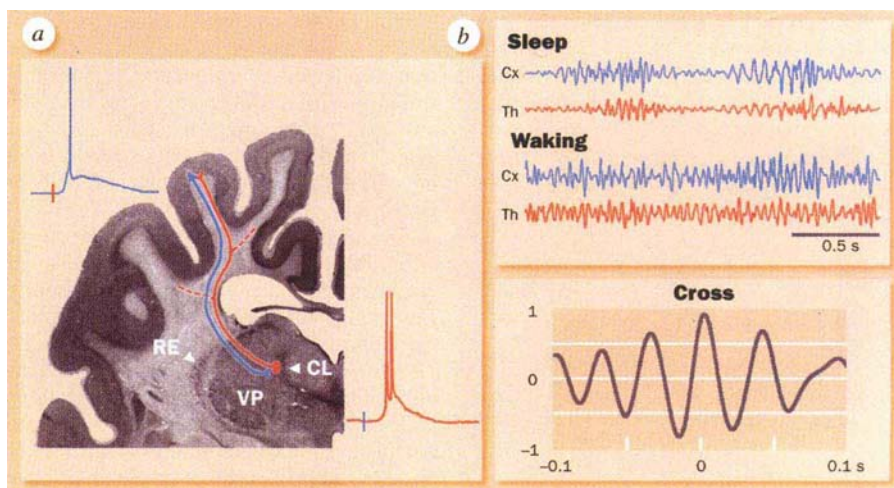
Mircea Steriade

BRAIN states of vigilance may be divided into three broad classes: wakefulness, resting sleep characterized by absence of consciousness, and rapid-eye-movement (REM) sleep during which dreams occur. These states are associated with distinct patterns of spontaneous electrical activity, as shown on the electroencephalogram (EEG). During resting sleep, the brains of both humans and animals generate low-frequency, high-amplitude waves; but during either REM sleep or wakefulness the brain is activated and the EEG gives way to high-frequency oscillations (typically 30–40 Hz) known as gamma oscillations. Activation is controlled in large part by a system that originates in the brainstem

and projects via synaptic relays in the thalamus to the cerebral cortex.

Understanding this pathway is thus central to understanding sleep and wakefulness, and the paper by Barth and MacDonald on page 78 of this issue¹ takes us closer to that goal. They show that a part of the thalamus known as the intralaminar nuclear complex is an essential component of the pathway.

The thalamus is the main source of input to the cerebral cortex, and it consists of many different nuclei. Some of these nuclei relay signals of a particular sensory modality to a specific cortical area; for instance, most cells in the medial geniculate nucleus of the thalamus are specific for



a, Coronal section through the cat brain showing the thalamus and cerebral cortex. Neurons in the centrolateral (CL) intralaminar thalamic nucleus (red) project to association suprasylvian cortex, exciting a pyramidal cell (blue) which in turn projects back to the thalamus. Projections from the intralaminar nucleus to many other cortical areas are not shown. Postsynaptic excitatory responses in both (thalamocortical and corticothalamic) directions are illustrated by means of intracellular recordings. RE and VP, reticular and ventroposterior thalamic nuclei. b, Field potential activities (filtered, 15–80 Hz) were recorded from cortical area 5 (Cx, blue) and thalamic intralaminar CL nucleus (Th, red) during resting sleep (S) and waking (W). During S, fast oscillations were interrupted periodically (due to cells' hyperpolarization related to a slow oscillation), whereas fast oscillations were uninterrupted during W. Cross-correlation (Cross) between cortical and thalamic activities during W shows synchronization within the frequency range of fast oscillations (around 28 Hz).

auditory stimuli and project exclusively to the auditory cortex. The thalamic intralaminar nuclei, however, receive inputs from many sensory modalities (including the highly arousing pain pathways), and project widely over many cortical areas with no discernible topography².

Importantly, thalamic intralaminar neurons are a major target for the brainstem reticular formation, a region known to be involved in triggering and maintaining a state of arousal. The reason why we can be awakened by a strong sensory stimulus is presumably because such a stimulus can activate large parts of the cerebral cortex via a circuit involving the brainstem reticular neurons that excite thalamic intralaminar neurons with cortical-projecting axons³ (see figure). The role of intralaminar nuclei in regulating arousal is underlined by the fact that their activity increases during a task requiring alertness and attention in humans⁴. Moreover, whereas damage to a particular sensory relay nucleus of the thalamus leads only to deficits in that particular sensory modality, bilateral destruction of the intralaminar nuclei in stroke patients leads to a prolonged state of lethargy or somnolence⁵.

How exactly do the intralaminar nuclei influence activity within the cortex? A special group of intralaminar neurons show a peculiar pattern of activity: during wakefulness and REM sleep, they fire rhythmic bursts of extremely rapid (1,000 Hz) spikes, with the bursts themselves occurring at about 20–40 Hz (ref. 6). Multisite, extra- and intracellular recordings from thalamus and cortex have shown that during an aroused state the thalamic gamma oscillation becomes synchronized with the oscillatory activity of the cortex⁷. (Although transient gamma oscillations also occur during resting sleep, they are interrupted by long periods of inhibition, whereas those that occur during full arousal are almost continuous; see figure.) These data support the hypothesis that the widespread appearance of fast waves over the cerebral cortex during waking and dreaming sleep is due to synchronization processes in thalamocortical systems through the intralaminar nuclei⁸.

There has been debate as to whether

the cortex⁹ or thalamus¹⁰ is the main source of the gamma oscillation. As Barth and MacDonald¹ show, the cortex alone can generate its own oscillation even in the absence of thalamic input from the posterior intralaminar nucleus. This result is probably due to the compensation by afferents from other parts of the intralaminar complex⁶ as well as from intracortical pathways oscillating within the same range of fast frequencies¹¹. In fact, gamma oscillations may arise in both the thalamus and cortex, as their neurons are equipped with intrinsic properties and network operations that underlie these fast rhythms.

So the new study¹ suggests a more subtle role for intralaminar nuclei; rather than driving the cortical oscillations, they are required to coordinate the oscillations between different cortical regions, in this case the primary and secondary auditory cortices. Surprisingly, electrical stimulation of the nearby medial geniculate nucleus, which normally conveys auditory signals to the cortex, abolishes the fast oscillations altogether. The significance of this observation is unclear, but it will be important to

determine whether abolition is due to activation of neurons within the auditory thalamus itself rather than other (as yet unidentified) fibres that may pass close to the site of electrical stimulation. One test will be to replicate the results using glutamate agonists, which excite cell bodies and dendrites without affecting passing axons.

The results of Barth and MacDonald reinforce the view that the intralaminar nuclei are essential in coordinating activity between several cortical areas, and may thus contribute to the formation of global perceptions of complex stimuli. We should now dig harder to understand at the cellular level how sensory signals (during wakefulness) and internally generated patterns of activity (such as pontogeniculo-occipital waves which occur during dreaming) can reset the spontaneously occurring fast oscillations and enhance their synchronization, and what role the intralaminar nuclei play in this process. □

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IMMUNOLOGY

A complement to host defence

Peter Henson

DEFENCE against infection is mediated first by recognition of the invading organism, and then by initiation of inflammation with the recruitment of circulating, motile, phagocytic and bactericidal white cells such as neutrophils to eliminate the invading organisms. This innate immunity is the first line of defence against infection, and there is much interest in its further role in directing or modifying deployment of the acquired immune response (that is, the second line of defence)¹.

The complement system is central to innate immunity. It is a highly regulated and complex set of interacting proteins, distributed between the blood plasma and cell surface, which can directly recognize and bind bacterial and fungal surfaces, leading to the killing and removal of the infectious organisms by phagocytes. The complement system also interacts with and enhances (literally complements) the acquired immune system by mediating the killing and removal process after the foreign organism has been recognized by antibody. As part of its mode of action in innate immunity, complement is also intimately involved in the process of inflammation—the complex and somewhat circumscribed response of different body tissues to injury from infections and a wide variety of other agents.

Using the power of gene deletion by homologous recombination, Höpken *et al.* (page 86 of this issue²) reveal a new level

of importance as well as complexity in the involvement of complement in both host defence and inflammation. The fifth component of complement, C5, is cleaved by C5 convertase, a multicomponent enzyme assembled from previously acting complement factors, to generate a fragment, C5a, which has various biological activities. These include smooth muscle contraction (C5a is also termed anaphylatoxin), effects on the microvasculature and chemotaxis of granulocytes leading to initiation of the acute inflammatory response.

Höpken *et al.* have deleted the receptor for C5a in mice, and show that these animals are not protected against infection with the bacterium *Pseudomonas aeruginosa*. By itself this is not surprising, because strains of mice having a natural deficiency of the parent molecule C5 are more susceptible to infection with a variety of organisms, including *Pseudomonas* and *Haemophilus*^{3–5}. The conclusion from this earlier work was that, in these infections, C5a serves primarily to attract neutrophils to the site to deal with the invading organisms with their well-known bactericidal and phagocytic properties. But the receptor-knockout animals of Höpken *et al.* showed increased accumulation of neutrophils in their lungs after *Pseudomonas* instillation compared with animals with normal C5a receptors, even though the bacteria were not killed and the animals went on to succumb to the infection. ▶

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How can this apparent paradox be explained? The first possibility that comes to mind is that neutrophils without C5a receptors have defective bactericidal and/or phagocytic properties, even if, because they are recruited in large numbers, they can still respond to some other chemotactic factor generated by the infection. But neutrophils in peritoneal exudate from the knockout mice were perfectly able to ingest and kill bacteria *in vitro*. Furthermore, following initiation of inflammatory responses in the peritoneum with the sterile 'irritant' glycogen, neutrophil accumu-

protein synthesis, or other products of the innate immune system. Intriguingly, neutrophil accumulation was usually reduced or delayed in the earlier studies with C5-deficient mice³⁻⁵, raising the possibility that other products of C5 cleavage, perhaps the terminal C5b-9 membrane attack complex (MAC), might contribute to the generation of chemotactic factors. This complex inserts into membranes and can both activate and lyse target cells⁶⁻⁸. Chemotactic agents that might result include interleukin-8 (IL-8) and leukotriene-B₄.

The differences in bacterial clearance

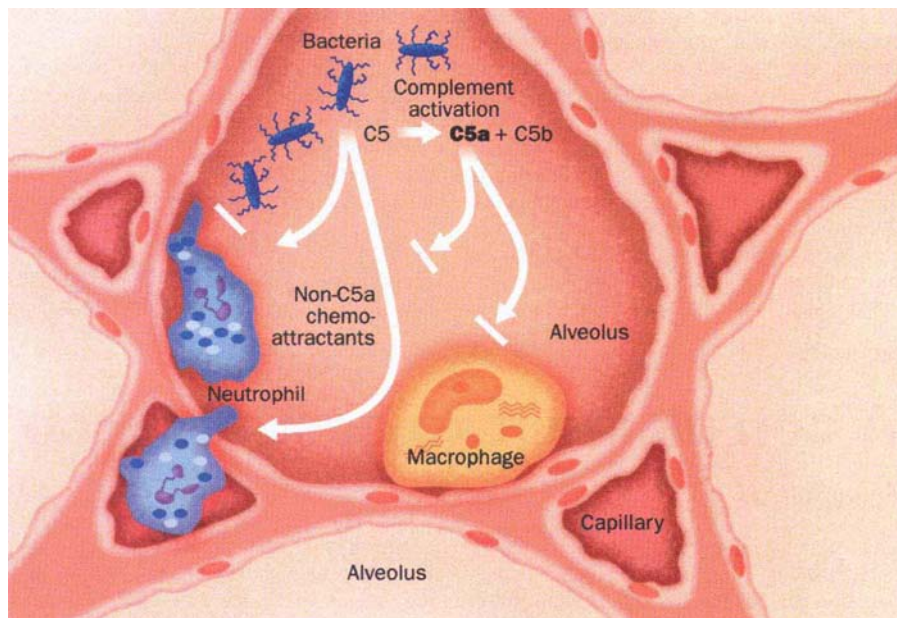
necrosis factor- α or platelet-activating factor) that act as priming agents for neutrophil bactericidal activity. Priming optimizes production of toxic (bactericidal) oxygen metabolites by neutrophils in a two-step process, in which one stimulus 'primes' the cells for more effective signal transduction by a second^{9,10}. Various combinations of agents, including chemotactic factors themselves, can serve as either priming or triggering stimuli.

The 'normal' bactericidal properties of C5a-receptor-deficient neutrophils *in vitro* might then not be reflected in comparably normal function in the lung, where C5a-generated co-stimuli could be essential to bring these cells up to their full potential. Alternatively, C5a may be a more complete activator than the other chemoattractants (formyl peptides, for example) involved in attracting the neutrophils, the latter being unable by themselves to initiate the full bactericidal properties of the cells.

Even these possibilities do not easily explain the unique responses of the lung compared with the peritoneum, however, and the presence of lung-specific inhibitors of microbicidal activity that are overcome by C5a-generated factors must be considered a possibility. On the other hand, neutrophil accumulation and permeability in the C5a-receptor-deficient lung was increased compared to normal. The most likely explanation is that this reflects the higher bacterial burden in these animals.

Whether the lung is representative of other mucosal surfaces or is unique remains to be seen. It is notable that microvascular responses in inflammation of the lung parenchyma (pulmonary circulation) are different from those involving tissues supplied by the bronchial or other systemic blood vessels¹¹; in consequence, C5a might have unique effects at the point of intersection between two classical components of the protective inflammatory response, the blood vessels and the phagocytes.

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In the normal lung, bacterial infection leads to activation of complement with generation of the chemoattractant C5a. This in turn attracts neutrophils from the capillaries and stimulates them to become bactericidal. In the C5a-receptor-knockout mice of Höpken *et al.*², alternative chemoattractants are still able to induce neutrophil accumulation, but the protective response is inadequate and the animals succumb to the infection.

lation was reduced, showing the expected dependency on complement and C5a-mediated chemotaxis.

If the C5a-receptor-knockout mice were infected intraperitoneally with *Pseudomonas*, on the other hand, neutrophil accumulation was neither reduced nor increased compared with normal animals. Even more importantly, the animals did not succumb to the infection. In other words, the receptor-deficient animals were perfectly capable of clearing *Pseudomonas* from the peritoneum.

At both sites, the chemotactic factors leading to neutrophil accumulation in response to *Pseudomonas* infection appear to be independent of C5a. This statement presupposes the presence of only one chemotactic receptor for C5a, the one that has been knocked out, which seems likely because neutrophils from knockout animals do not respond to C5a *in vitro*. Alternative chemotactic candidates could include products from the bacteria themselves, such as the *n*-formyl peptides that result from bacterial but not eukaryotic

between the two tissues suggest that C5a and its receptor have a protective function in the lung that is not operative in the peritoneum. But why are the neutrophils that do accumulate in the lung not effective? Phagocytosis of bacteria by neutrophils requires opsonization, generally by antibody and/or the C3 component of the complement system. C5a is also known to increase vascular permeability (another way in which it contributes to inflammation), so it could enhance neutrophil function by supplying plasma proteins to the alveolar surface of the normal lung. However, permeability was not prevented in the C5a-receptor-knockout mice.

These observations might imply that neutrophils do not play the prime defensive role *in vivo* normally ascribed to them. But it is more likely that the pulmonary environment requires other factors for optimal neutrophil function that are produced by secondary actions of C5a. For example, macrophages respond to C5a and synthesize a variety of cytokines and other molecules (such as tumour

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Frank Whittle (1907–96)

AIR Commodore Sir Frank Whittle, British pioneer of the jet engine, died on 8 August in the United States, where he had lived for the past 20 years. As one of the first to link the idea of the gas turbine with the concept of jet propulsion, Whittle was unique in persevering and bringing his invention to fruition and into service. He stands alongside the most creative of British engineers, such as Watt, Stephenson and Parsons, whose names are linked with successive stages in the development of steam power. His simple single-shaft centrifugal turbojet was responsible for introducing the jet engine to the aviation industry virtually worldwide.

Whittle joined the Royal Air Force as a Boy Apprentice in 1923, and by the early 1930s he had become one of its top pilots. In 1934, the Air Ministry arranged for him to go to Cambridge University. In 1936, six years after he had patented the first jet engine, a small company was formed, Power Jets Ltd, with which he was associated until 1946. He retired from the RAF on health grounds in 1948, having reached the rank of Air Commodore. Between 1961 and 1970, Whittle was a consultant to Bristol Siddeley Engines and Rolls-Royce, and even at the age of 80 he was working on his own designs for supersonic aircraft.

He was a man of many parts; pilot, inventor and engineer. He was both a professional test pilot on sea-planes and a star turn at the pre-war Hendon RAF displays. As an inventor, he filed his first jet engine patent in 1930, followed by many more covering a range of jet and other developments.

As an engineer, he was notable for the way in which he approached the jet engine and other technical developments from first principles — even before he had the benefit of an academic qualification. His eminent contemporary at Rolls-Royce, Dr Stanley Hooker, described Whittle as having an unrivalled grasp of the fundamentals of thermodynamics and aerodynamics, and that he had laid down the performance of the jet engine "with the precision of Newton", an achievement about which he was characteristically modest.

Whittle's approach differed from that

of other jet engine inventors in Europe and the United States, such as Lysholm (Sweden) and Hobbs (United States). His emphasis was on simplicity of design, and in seeking to use the gas turbine where it was most efficient — at high speed at high altitude. His contemporaries in industry and government research establishments made the error of opting for complicated configurations

IMAGE
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"There is no doubt that the aircraft gas turbine is going to have profound effects on future aircraft development. I predict that in from five to ten years' time we shall see the piston engine displaced in all new aircraft except the light aeroplane." F. Whittle, *Nature* **157**, 391–395 (1946).

in the pursuit of a performance competitive with that of the piston aero engine. None of these concepts proved successful.

The configuration chosen by Whittle was the single-shaft turbojet with single-stage centrifugal compressor and axial turbine. This arrangement became the hallmark of Whittle's work, and launched the jet engine industry around the world. The sole exception was in wartime Germany, where the more complicated but potentially more efficient axial compressor was adopted following the success of a centrifugal turbojet developed by Hans von Ohain at Heinkel. But all German work was prohibited by the Allies for a decade after the war, leaving the field clear for derivatives of Whittle's engines.

The historic year for Whittle was 1936. He graduated from Cambridge University with a first-class honours

Mechanical Sciences Tripes; with the help of two ex-RAF colleagues, Power Jets Ltd was formed to develop his ideas; and British Thomson-Houston Co. Ltd was contracted to manufacture his WU (Whittle Unit) experimental engine, which first ran in April 1937.

From then on, Whittle had to struggle every step of the way against an uncooperative Air Ministry and Ministry of Aircraft Production. Even worse was the attitude of the Rover Co. Ltd, which had been selected to produce the W2B service engine. This, and the frustration of not receiving due recognition for his successes or support for his continuing efforts, steadily eroded Whittle's health. Official incompetence also contributed to the failure of the Whittle-powered Gloster Meteor fighter to be ready in time to have more than a passing involvement in the Second World War.

Although in 1946 he resigned from Power Jets a disappointed man, Whittle had achieved much in the decade since his first engine ran. During the war, details of his work had been passed to General Electric in the United States, where his centrifugal turbojet launched the US jet engine industry. At the same time in Britain, de Havilland developed its own version of the Whittle turbojet; and in 1943, Rolls-Royce took over the W2B programme from Rover, and started its own series of Whittle-derived engines.

Through these three companies, Whittle's ideas and technology spread worldwide. Whittle-derived engines were built not only in Britain and the United States, but also under licence in Australia, Belgium, Canada, France, Italy, Sweden and Switzerland. Significantly, Rolls-Royce also sold engines to the Soviet Union, where they were reverse-engineered and put into mass production. Later, they were licensed by the Soviets to Czechoslovakia, Poland and China. Something approaching 100,000 Whittle engines were built, powering a wide variety of combat, experimental and commercial aircraft. Few inventors have left so tangible a legacy.

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Dangers hang over dependency

Peter Francis

MONTSERRAT is erupting. Starting in late July, the Soufriere Hills volcano which dominates the southern part of the island has become considerably more active. Sustained periods of low-frequency, high-amplitude tremor have been recorded by seismographs, implying the intrusion of new magma, three to five kilometres beneath the summit. They also cause continuous rock falls lasting for several hours, such as the one that produced the dense curtain of ash shown here (Fig. 1).

Occasionally much larger 'pyroclastic flows' of hot rock and gases have been produced. Several of them have travelled along the valley of the Tar River as far as the sea, building a new delta that extends 400 metres into the sea. Dust clouds rise convectively from the pyroclastic flows and reach an altitude of about 10 kilometres, triggering displays of thunder

and lightning, and presenting a hazard to aircraft using the many routes that criss-cross the Caribbean.

The reawakening of the Soufrière Hills volcano last year after 400 years of inactivity precipitated a crisis for this small British Dependent Territory. Thousands of people have been moved from the southern part of the island, including the capital town of Plymouth, only four kilometres from the crater.

Since September 1995, the volcanic activity on Montserrat has been domi-

nated by extrusions of andesitic lava into the summit crater at a rate of about 100,000 m³ per day, forming a new dome complex. This is a unique opportunity for many scientists (from the University of the West Indies and from Britain) to study the evolution of a lava dome at close quarters using a wide range of geophysical and



FIG. 2 Convective ash columns above the volcano on 29 July. Although resembling an explosive eruption column, there was no true explosive activity, and no open vent. The ash column was made of dust from rocks pulverized in avalanches.

geochemical techniques. An important problem, for both scientific and practical reasons, is why the magma is depleted in volatiles, with a low ratio of SO₂ to HCl. It is the volatile content of magmas that powers dangerously explosive eruptions.

Intermittently, the dome becomes so steep that portions break away to become avalanches of hot rocks, variously termed glowing avalanches, *nuées ardentes* or pyroclastic flows. These avalanches in turn generate tall, convective ash clouds (Fig. 2). This sort of activity is well known as a volcanic hazard in the West Indies, having first been seen on the neighbouring island of Martinique during the catastrophic 1902–1905 eruption of Mt. Pelée which destroyed the town of St Pierre.

Ever since the dome began to grow, the concern has been that it might eventually overtop the western rim of the existing crater wall, allowing pyroclastic flows to descend towards Plymouth. Although part of the dome did rise above the wall on that side, the most recent activity has been concentrated on the north-east side of the dome, so recent pyroclastic flows have all followed the path of earlier flows down the Tar River. But the message from Mt. Pelée and elsewhere is that eruptions of this sort can continue for months or even years, and parts of southern Montserrat could be under threat for a long time. □

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Surprising damp

BUILDERS and householders, especially in rain-soaked Britain, are terrified of rising damp. A wall of porous bricks based in wet soil is soon saturated with water, which rises through the capillary network of its pores. It softens plaster, peels paint and rots woodwork attached to the wall. One answer is the electro-osmotic damp-proof course. This keeps the wall a volt or so positive with respect to the ground. The potential difference generates an electro-osmotic pressure in the pores of the brickwork, which opposes upward capillary flow. But installing the system requires some care. If it is accidentally given the reverse polarity, it will suck the damp upwards with extra force.

In this connection, Daedalus notes that plants depend entirely on rising damp. They take in water through their roots, pump it up through their stems and branches, and expel it as vapour from their leaves. In clean and well soaked soil, they flourish mightily. But in salty soil, they have problems.

To extract water from salt solution, the roots of the plant have to suck against an osmotic pressure of maybe many atmospheres. They have to generate an even greater extractive osmotic pressure of their own, taxing the metabolic resources of the plant.

So Daedalus is devising a reverse-polarity electro-osmotic pumping system for plants, to help their damp to rise. The smaller the pores in such a system, the greater the resulting pressure. Daedalus estimates that across the nanometre-sized pores of a semi-permeable root membrane, a few tens of volts could generate many hundreds of atmospheres of extractive osmotic pressure. DREADCO biophysicists are now tying electrodes around the stems of various vegetables, bushes and trees, and burying counter-electrodes in the soil nearby. With the right strength and polarity of applied voltage, the plants should grow with accelerated vigour even in salty or contaminated soil. When the system has been perfected, DREADCO will sell it to the world's farmers.

At last the creeping pollution of the world's water tables and irrigation systems will no longer matter. Aided by electric power, crops will flourish in industrial effluent, salt marshes, maybe even in the sea itself. With a high enough voltage, a plant might even extract water from the humidity of the air. It would then become a sort of electrically powered living dehumidifier, condensing water from the air onto its roots, evaporating it back from its leaves, and needing no water or soil at all. The industrialization of agriculture would be complete.

David Jones



FIG. 1 Dust clouds, drifting many kilometres downwind of the rock falls that produced them.

Perception of music by infants

SIR — The origins of the perception of consonance and dissonance in music are a matter of debate, well-illustrated by the difficulty in assimilating contemporary atonal music, where dissonance is prominent. Here we suggest that infants are prepared to find consonance perceptually more attractive than dissonance.

Consonance and dissonance are concepts that refer to the pleasingness of two

music theorists⁸.

By exposing young infants to consonant and dissonant music, we tested the hypothesis of an innate bias favouring consonance over dissonance. We presented a sample of 32 4-month-old infants (16 males, 16 females) with two different, unfamiliar melodies of 35-second duration in a consonant and a dissonant version for a total of four trials. We created

to the dissonant versions of each melody (see figure). We believe these data suggest that the infants prefer the consonant to the dissonant versions of the melodies.

Two main factors control duration of visual fixation in the infant: preference and discrepancy¹¹. The dissonant versions of the melodies deviate from what the infant is used to hearing and therefore can be considered discrepant events. However, because the infants do not show longer fixations to the dissonant stimuli, it is fair to assume that it is preference, and not discrepancy, controlling the infants' fixation time. Motor activity reflects arousal, but arousal can be pleasant or distressed. Because the dissonant versions elicited increased motor activity but less fixation time, motor activity probably reflects a distressed state rather than a pleasant one.

There is no relation between infants' previous exposure to music (as assessed by a questionnaire) and their behaviour in the experiment. Although less extreme forms of consonance/dissonance might be subject to cultural influence, we suggest that the human infant may possess a biological preparedness that makes consonance perceptually more attractive than dissonance.

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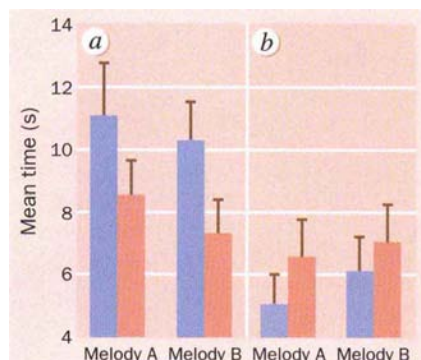
Protrusible eyes; retractable noses

SIR — The closest thing to the protrusible eyes in the vertebrate caecilians described by O'Reilly *et al.*¹, are the tentacles of snails and slugs. The posterior tentacles of terrestrial gastropods (Pulmonata, Stylommatophora) similarly house an eye and an olfactory organ, and they are fully retractable. Terrestrial pulmonates are strongly dependent on environmental water, so, like the caecilians, they are essentially amphibian.

In contrast to the stylommatophores (eyes at the tentacular tip), freshwater pulmonate species (Basommatophora) have their eyes in the head, at the bases of the tentacles. Thus, it can be argued that the retractable tentacle is an evolutionarily stable, if functionally tentative, device for exploration of the terrestrial environment, with obvious advantages for accessing relevant chemical and visual stimuli.

A matter of further interest is the

a, Mean total visual fixation time to music source during the consonant and dissonant versions of two melodies. Infants displayed significantly longer fixation times during the consonant (blue boxes) compared to the dissonant (red boxes) versions of the melodies (Repeated measures analysis of variance on square-root of data: $F(1,24) = 11.50$; $P < 0.005$). **b**, Mean time of motor movement during the consonant and dissonant versions of the two melodies. Infants showed less motor activity during the consonant compared to the dissonant versions of the melodies (Statistics as above: $F(1,24) = 6.64$; $P < 0.02$). Full details of methodology and other results are available on request from M. R. Z.



or more frequencies occurring together. The most basic example is an interval, which is the difference in pitch (frequency of vibration) between two tones. Some intervals are considered dissonant, others consonant. Although Helmholtz and other psychophysicists believed that consonance judgments rested on inborn properties of the auditory system¹⁻⁴, biological preparedness has never been demonstrated. Psychoacoustic laws, as formulated by Helmholtz and others, could reflect acquired, rather than inborn, mechanisms of auditory processing. Thus, it has been argued repeatedly that consonance judgments are acquired through exposure to the music of a particular culture^{5,6}. Starting with Schönberg⁷, this view has become prominent among modern composers and

the stimuli using a computer program that controlled an attached music synthesizer. We manipulated consonance and dissonance by composing the dissonant version in parallel minor seconds, the consonant version in parallel thirds using only two (synthetic) voices (Hz range of upper voice: 329.63–698.46). In this range, the minor second is consistently rated as the most dissonant interval, whereas the third is experienced as consonant^{2,9,10}.

We placed each subject in an infant seat facing a speaker baffle at an angle of 45° to the infant's right and covered with an attractive pattern of concentric circles. We recorded the infant's behaviour with a video camera. There was a 35-second quiet baseline followed by the four melodies, with an interval between each melody of 8 seconds. The music was delivered at a sound pressure level (SPL) of 60 dB. We assigned each infant to one of four different orders of stimulus presentation.

We coded the video record for fixation time (*a* in the figure) and motor activity (*b*). Fixation time is total time looking at the pattern covering the speaker. Motor activity is the total duration of flexion of one or both arms and legs of at least 60°, and movement of one or both arms and legs by at least 60° to the right or left and more than 2.5 cm in vertical direction. (Inter-coder reliability is greater than 0.90 for both variables.)

Infants look significantly longer at the music source and show less motor activity when hearing the consonant as compared

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specialization of the snail's brain that has been attributed to the appearance of the retractable nose. As Van Mol² was the first to point out, a prominent procerebral lobe is present in all stylommatophore species but in no basommatophore species. Could the brain of caecilians have a comparable adaptation? The possibility is suggested by numerous analogies that are evident in the olfactory systems of snails and vertebrates. Convergent features include the cellular organization of the sensory epithelium, the presence of synaptic glomeruli and the generation of oscillating field potentials^{3,4}. Given that snails and caecilians seem to be the only ani-

mals with mobile and retractable eye/nose structures, there might be curiosities to discover in the caecilian brain, especially in those regions that receive afference from the tentacle.

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Transposable element in fish

SIR — Several transposable elements of the terminal-inverted-repeat class have been described in vertebrate genomes¹. Some of them are thought to be active because of their presence in gene regions or polymorphic situations. However, demonstration of *de novo* excision or insertion of such elements has hitherto not been described to our knowledge. We have identified a novel terminal-inverted-repeat class transposable element in the medaka fish *Oryzias latipes*, and have detected its excision during embryogenesis.

The *i*¹ / *i*¹ medaka fish genotype is associated with a complete albino phenotype², as the *i* allele is a defective tyrosinase gene³. We isolated another allele at the same locus, *i*⁴, from a commercial breed-

ing population: *i*⁴ / *i*⁴ fish have a quasi-albino phenotype. The *i*⁴ allele is recessive to the wild-type allele *i*⁺. A description of *i*¹ and *i*⁴ is available on the World-Wide Web at the Medakafish home page (<http://biol1.bio.nagoya-u.ac.jp:8000/>). Cloning and sequencing of the tyrosinase gene region of an *i*⁴ / *i*⁴ fish reveals that the fifth exon contains a 4.7-kilobase (kb) DNA insertion. We believe that the insertion sequence (*a* in the figure) is a terminal-inverted-repeat transposable element because: (1) it has inverted repeats at its termini; (2) there is duplication of a segment of the host chromosome; and (3) it is present as multiple copies. We have called this transposable element *Tol2*, and the particular copy found in the tyrosinase gene *Tol2-tyr*.

About 10 copies are present in the diploid genome.

Tol2 contains 4 open reading frames with amino-acid sequence similarity to *Ac* of maize⁴, *hobo* of *Drosophila*⁵ and *Tam3* of snapdragon⁶ (see Supplementary Information at *Nature's* website, <http://www.nature.com>). We propose that these elements have diverged from a single ancestor⁷.

Transposable elements of the terminal-inverted-repeat class are thought to move in a cut-and-paste fashion. To detect excision of *Tol2* during embryogenesis, we conducted polymerase chain reaction (PCR) using a pair of primers which encompass *Tol2-tyr* and are 0.5-kb apart on the wild-type tyrosinase gene⁸. We used genomic DNAs of 5-day-old albino embryos as templates. We examined 60 embryos individually, and all show 5.2-kb

products. In addition, we observed 0.5-kb fragments from 8 of the 60 embryos examined (see Supplementary Information). We cloned and sequenced these 0.5-kb fragments and grouped the sequences obtained into six types, all featuring total, or near-total, loss of the *Tol2-tyr* sequence (*b* in the figure). Type 5 is identical to the wild-type fish product. In the other five types, some nucleotides of the target site duplication or a *Tol2* terminal region are retained. Thus our PCR analysis provides evidence for *de novo* excision of *Tol2*, with good but not absolute precision. Excision with some nucleotides left behind has also been observed for *Ac*⁹ and other terminal-inverted-repeat class transposable elements.

Whether *Tol2-tyr* itself is an autonomous member is not yet known. It is evident from our results, however, that an autonomous member is present somewhere in the genome. *Tol2* is thus a unique tool for establishing a gene tagging system in fish, and possibly also in other vertebrate species.

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Miho Suzuki

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Yoshitaka Bessho

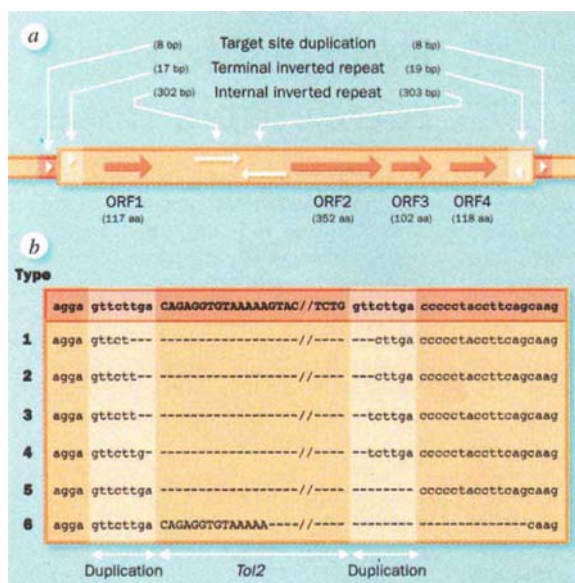
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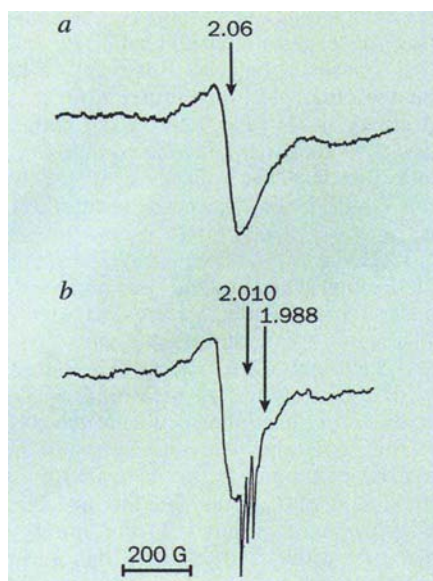
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Dynamics of haemoglobin

SIR — Jia *et al.* describe a possible physiological role of nitrosylated haemoglobin, postulating a dynamic cycle in which oxygenated haemoglobin in arterial blood is S-nitrosylated and the NO group is released to bind to the haem of deoxygenated haemoglobin during arterial-venous transit¹. Thus the authors predict, citing ref. 2 as an example, that haemoglobin with haem-bound NO may not be observed in arterial blood but may be in venous blood. They contend also that the anticipated normal levels of haem-nitrosylated haemoglobin *in vivo* are too low to be detected by direct measurements (for example, electron paramagnetic resonance (EPR) spectroscopy).



Characterization of *Tol2*. *a*, Structure. Total length, 4,681 bp (GenBank accession number D84375). *b*, Excision footprints. Nucleotides of *Tol2* and the tyrosinase gene are shown in upper and lower case, respectively. Dash, no nucleotide occupation. The boxed sequence is from the genomic clone of the tyrosinase gene region that contains *Tol2*.



Low-temperature (77 K) EPR spectra of arterial (a) and venous (b) blood collected from a male Sprague-Dawley rat 60 min after exposure to air-blast overpressure (62.2 kPa). This level of air blast overpressure induces oxidative stress as evidenced by depletion of pulmonary antioxidants (total antioxidant reserve decreased by 300%, vitamin C by 50%, vitamin E by 30%) and an increased level of lipid peroxidation products (by 200%). a, The spectrum contains a broad feature at $g = 2.06$ assigned to ceruloplasmin⁶. b, The spectrum contains two species: the broad ceruloplasmin signal ($g = 2.06$) and a characteristic three-line signal of α -Hb[FeII]NO (T-state) with hyperfine structure (splitting constant (A_z) for ^{14}N of 16.5 G) centred at $g = 2.010$ ⁴. Importantly, the signal from β -Hb[FeII]NO, known to arise from hexacoordinated nitrosyl haem-iron complexes (R-state) (trough at $g = 1.988$ ⁴), is not detectable in the spectrum. Hence, no more than two NO molecules are bound to haem, and only to α -haems. Scale bar, 200 G.

We used a compressed air-driven shock tube to deliver the blast to rats as described previously³. Blood was collected from sodium pentobarbital anaesthetized animals and venous blood obtained from the portal vein. Arterial blood was obtained by cardiac puncture. To prepare tissue samples of constant volume and geometry for EPR measurements, we filled a small aluminium tube with blood and immediately immersed it in liquid N_2 . The resulting tissue casts were consistent in volume and shape. We kept samples at 77 K during the EPR experiments, which we performed with a JEOL-RE1X (X-band, 100-kHz modulation) spectrometer.

We report here that haem-bound NO (Hb[FeII]NO) becomes detectable in venous blood when animals are exposed to oxidative stress. Hb[FeII]NO was detected in venous blood of rats by low-temperature EPR spectroscopy after the animals were exposed to air-blast overpressure (a model previously demonstrated to produce extensive oxidative stress *in vivo*³). The Hb[FeII]NO pentacoordinated complex was characterized by a pronounced three-line spectrum (see figure). This spectrum is typical of α -subunit haem-bound NO (T-state)⁴ without any contribution from the characteristic signal of the β -subunit Hb[FeII]NO-producing R-state (Fig. 1). In accord with the predictions of Jia *et al.*, we did not observe any EPR signals for haem-bound NO in venous or arterial blood from unstressed animals.

We previously reported similar characteristic EPR signals of the T-state of Hb[FeII]NO (A_z for ^{14}N of 16.6 G detected in venous (but not arterial) blood from CBA mice infected with a pathogenic strain of influenza virus A/Victoria/35/72 (H3N2) (another *in vivo* model known to produce oxidative stress)⁵. Consistent with our findings are those of Hall *et al.*⁶, who reported EPR signals of the T-state of Hb[FeII]NO in venous blood from rats exposed to hyperthermia, and of Akaike *et al.*⁷, supporting our findings in mice.

According to Jia *et al.*, nitrosylation of Cys β 93 in oxygenated haemoglobin is due to an increase in reactivity of this thiol — a result of improved access of NO to it via allosteric transition in the portion of the protein it occupies when O_2 binds to the β -haem. This is a compelling argument

and may explain the conserved nature of Cys β 93 in haemoglobins across species. In the rat, tetrameric haemoglobin has four reactive β -chain Cys residues. Importantly, Jia *et al.* reported stoichiometry of about three sulphur-bound NO molecules per tetramer of S-nitrosylated rat haemoglobin (SNO-Hb). This suggests that no more than three NO may become available for interaction with the haems of deoxygenated haemoglobin in venous blood, if indeed SNO-Hb is the only source of NO.

Our EPR data suggest that no more than two NO molecules interact with haem in deoxygenated haemoglobin in venous blood *in vivo* to form Hb[FeII]NO, and that the haems affected are in the α -chains. The admixture of the R-state of Hb[FeII]NO would be discernable in the EPR spectrum if NO was bound to either of the β -haems of haemoglobin. In fact, this R-state of Hb[FeII]NO can be easily detected in the presence of excess NO.

Our results and those of others described here confirm the hypothesis of Jia *et al.* and further extend it by showing that not more than two of the three S-NOs liberated from oxygenated haemoglobin bind to the α -haems of deoxygenated haemoglobin. Thus, in the NO binding-releasing-inactivation cycle proposed by Jia *et al.*, one NO must remain bound to one of the cysteines in rat haemoglobin. From their results, it seems likely that this NO is bound to one of the two Cys β 125 residues, thiols known to be reactive toward electrophiles that have no counterpart in human haemoglobin (β 125 in human haemoglobin is

proline) but have been noted in many rodents. Have rodents developed this additional reactive thiol in their β -chains as an adaptation to stresses unencountered by humans, or is its presence indicative of different methods for handling erythrocytic NO?

Thus, the report of Jia *et al.* leads to many additional questions other than those originally posed, among them the following. What are the roles of the two haem-bound NO molecules? How do they arrive at α -haem from Cys β 93 (or Cys β 125)? In both rat and human haemoglobin, the only α -chain thiol is Cys α 104, a buried residue generally considered to be unreactive towards electrophiles. Interestingly, Perutz's crystal structures of human deoxygenated haemoglobin show Cys α 104 and Cys β 93 to be nearly equidistant (about 13 Å) from their respective haems⁸. Is Cys α 104 also involved in the dynamics of the two haem-bound NO molecules? What is the fate of the two haem-bound NO molecules during the venous-arterial transit? Perhaps they undergo oxidation (thus eliminating excessive NO) and cause formation of methaemoglobin, which is in turn reduced by erythrocytic methaemoglobin reductase? What is then the fate of the third S-NO? Does it remain an S-NO or is it transferred inter- or intramolecularly to the next available unliganded α haem, perhaps via Cys α 104? If so, is this sulphur-bound NO used only for delivery (by SNO/SH exchange) and used by contracting endothelial surfaces, as suggested by Jia *et al.*?

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references.

Ball lightning an electromagnetic knot?

SIR — Here we propose a model of ball lightning, a rare and beautiful phenomenon, so far unexplained. Usually, it appears as a flaming ball, most frequently red, orange or bright white, green or blue in some rarer cases, its diameter being typically 10–30 cm, although some examples of a few centimetres or of several metres have also been observed. It moves in an unpredictable way and disappears after several seconds, sometimes smoothly, occasionally with an explosion^{1,2}.

Several different theoretical explanations of this phenomenon have been proposed, based on microwaves, combustion, electrical or nuclear processes, or new states of matter — including the opinion that they are no more than optical illusions. No explanation is generally accepted.

Here we propose a solution based on the idea of electromagnetic knot^{3,4}, an electromagnetic field in which any pair of magnetic lines or any pair of electric lines form a link — a pair of linked curves (Fig. 1). Each knot is characterized by two integers n_m and n_e , the corresponding magnetic and electric linking numbers (the number of times that each curve crosses a surface bounded by the other). M. Berry (personal communication) suggested that we use them to try to model ball lightning (see also ref. 5).

For simplicity, we will consider here only knots of magnetic type, with no electric field. According to the method explained in refs 3 and 4, we obtain the following magnetic field whose lines are linked $n_m = n$ times

$$\mathbf{B} = \frac{4\sqrt{a}}{\pi L^2(1+R^2)^3} [2(Y-nXZ), -2(X+nYZ), n(X^2+Y^2-Z^2-1)] \quad (1)$$

where $(X, Y, Z) = (x, y, z)/L$, $R^2 = (X^2 + Y^2 + Z^2) = r^2/L^2$, L being a length which sets the space scale and a a normalizing constant.

The linking number n is related to the

so called magnetic helicity^{6,7} h as

$$h = \int_{R^3} \mathbf{A} \cdot \mathbf{B} d^3x = na \quad (2)$$

where $\mathbf{A}$ is the vector potential. This helicity does not depend on L and is a constant of the motion if there is no resistivity (if the conductivity is infinite).

If a magnetic field is coupled to an incompressible plasma that is hot enough, the magnetohydrodynamical approximation, in which the conductivity σ is assumed to be infinite, can be used. The motion is described by the Navier–Stokes equation coupled to the Maxwell equation for the magnetic field. A solution of this system is given by $\mathbf{v} = \pm \mathbf{B}/\sqrt{\rho}$, $p + B^2/2 = \text{constant}$, $\mathbf{v}$, ρ and p being the velocity, the density and the pressure of the plasma. These states, the streamlines of which are also linked, are not really stationary, as its energy

$$E = \int (\rho v^2/2 + B^2/2) d^3x = (n^2 + 1)a/L \quad (3)$$

decreases by increasing the length L (the mean square radius of the energy distribution). Because infinite conductivity implies very high temperature, the knot radiates strongly according to the Stefan–Boltzmann law, which is why it shines and has a flaming aspect. If the knot radiates the same amount as a sphere of radius L , its energy loss is $dE/dt = -\sigma^* 4\pi^2 L^2 T^4$, where σ^* is the Stefan constant and T the temperature, this forces the ball to decrease its energy by increas-

ing L according to equation (3). Assuming that the expansion is adiabatic and that the plasma behaves as an ideal monoatomic gas, its temperature must decrease as $T = \beta L^{-2}$, where β is a certain constant coefficient. From equation (3) and the last two relations, it follows that $L = L_0(1+t/\tau_n)^{1/5}$, where the characteristic time is $\tau_n = (n^2 + 1)\tau_0 = (n^2 + 1)L_0^5 a / (20\pi^2 \sigma^* \beta^4)$. Consequently, the radius of the ball increases, the magnetic field goes to zero, the temperature decreases as $T = T_0(1+t/\tau_n)^{-2/5}$, the plasma cools down and the brightness is reduced. The stability of the structure, as measured by the slowness of the cooling, is due to the constraint imposed by the conservation of the helicity equation (2), or equivalently by the linking of the lines. The linking of the knot keeps the plasma hot for a time that increases with n and is longer than for an unlinked system.

We have assumed that the initial temperature is high enough, say above 30,000 K, to imply infinite conductivity. If σ could remain infinite, the expansion would continue for ever as $(1+t/\tau_n)^{1/5}$. But the conductivity decreases together with the temperature, bringing two new effects into play: first, an additional energy loss by Joule effect which accelerates the cooling; and second, loss of helicity conservation. This leads to the destruction of the knot.

Thus, a magnetic knot formed by a lightning in a storm, where the air is ionized and the temperature very high (30,000 K at least), first radiates energy according to the Stefan–Boltzmann law. Insofar as the conductivity can be considered as infinite, the conservation of helicity constrains the decay of the ball, which loses energy by slow expansion as $(1+t/\tau_n)^{1/5}$. This produces a reduction of its temperature and a corresponding increase of the resistivity, which in turn produces helicity loss and dissipation of the structure.

To design an experiment to test this model in ball lightning is not easy for obvious reasons. There are two possible laboratory settings: tokamaks⁸ and devices constructed to produce fireballs⁹. Thus it may be possible to test the ideas proposed here in an appropriate experiment.

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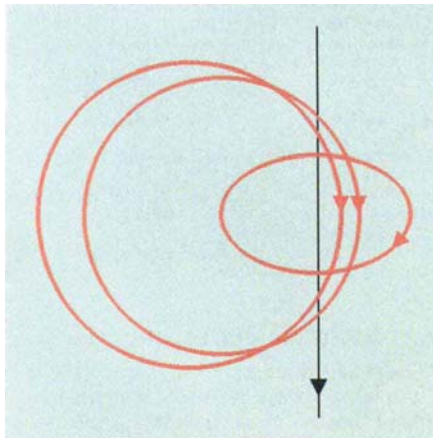


FIG. 1 Schematic representation of the magnetic lines in the case $n=1$.

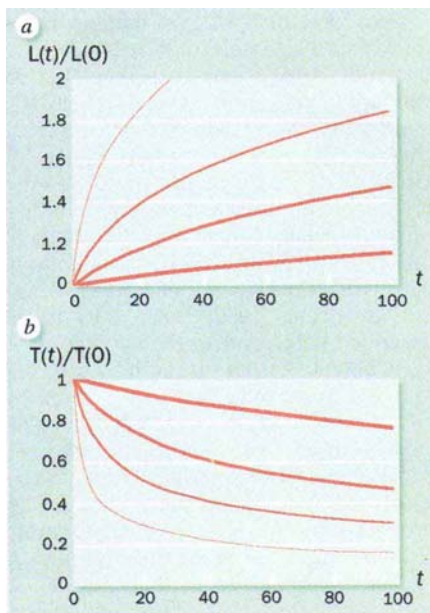


FIG. 2 a, $L(t)/L_0$ and b, temperature $T(t)/T_0$ versus time in units of τ_0 for infinite conductivity and for the linking numbers $n=0, 2, 4$ and 10 (in increasing order of thickness).

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Nervous dispositions

William Milberg

The Neuroscientist: A Review Journal Bridging Neurobiology, Neurology and Psychiatry. Editor Stephen G. Waxman. *Williams and Wilkins*. 6/yr. USA \$214, elsewhere \$239 (institutional); USA \$107, elsewhere \$132 (personal); USA \$59, elsewhere \$84 (student).

Journal of the International Neuropsychological Society. Editor-in-chief Igor Grant. *Cambridge University Press*. 6/yr. USA, Canada and Mexico \$160, elsewhere £112 (institutional); USA, Canada and Mexico \$80, elsewhere £61 (personal).

European Journal of Neurology: The Official Journal of the European Federation of Neurological Societies. Editor François Boller. *Rapid Science*. 6/yr. \$365, £215 (institutional); \$160, £95 (personal).

WHEN I was an eager graduate student in neuropsychology in the mid-1970s, I was under the impression that through consistent nightly reading of a few key journals one could keep up with the most important developments in behavioural neurology, basic neuropsychology and even the fundamental neurosciences. I was able to hold on to what was even then a quixotic overestimation of my own diligence because there were only about a dozen journals to worry about that contained 'brain', 'neuro' or 'cortex' in their titles.

Today there are at least a hundred such periodicals, so even the most compulsive, manic scholars of impeccable diligence could not hope to keep up with advances outside a few of the many new disciplines that have emerged in the neurosciences in the past two decades. This problem is particularly acute for such increasingly neuroscientific clinical disciplines as neurology, psychiatry and neuropsychology: each of these areas has spawned a clinical literature that in some cases has taken on a life happily independent of the concerns and issues of the basic sciences. Any publication that could help today's specialized investigators and practising clinicians to stay in contact with theoretical and empirical progress in the basic and clinical brain sciences would be surely welcome.

These three new journals seem to share the goal of providing both scientists and clinicians with a contact point encompassing a broad spread of topics ranging from the molecular genetics of neurological disease to evaluations of techniques for the assessment of dementia. Of the three, *The Neuroscientist*, which uniquely is a journal consisting entirely of critical review articles, is perhaps the most successful in presenting the specialist with an opportunity to survey areas that might not otherwise be easily accessible. Each issue contains a "Comments" section consisting of several brief items or abstracts of recently published results, "Neurosciences Updates" and "Progress" sections presenting medium-length reviews of topics of

emerging interest such as molecular neurogenetics, and a "Reviews" section containing longer articles on topics in the basic and clinical neurosciences. Personally, I enjoyed 'catching-up' on the role of the neurotransmitter GABA in mood disorders and on recent advances in the molecular biology of neural plasticity, topics that would otherwise be too far from my interest in cognitive and clinical neuropsychology.

This well-produced journal is replete with finely reproduced black-and-white photographs and well-constructed tables and graphs. Even though it does not contain peer-reviewed papers of original scientific data, it should usefully occupy a niche in institutional libraries and be a worthwhile addition to many personal libraries.

European Journal of Neurology (EJN) and *Journal of the International Neuropsychological Society (JINS)* are also designed to reach a broad audience of clinicians and scientists, in two different but related fields. Both have several features in common: they are flagship publications of large professional or scholarly societies; they contain forums for both empirical and review papers; they give authors the

opportunity to publish peer-reviewed results relatively rapidly (about three months); and they present an assortment of fairly high-quality papers on a variety of clinically driven topics.

For clinical neuropsychologists, *JINS* achieves its goal of allowing them to keep abreast of issues in basic experimental neuropsychology. It contains several departments unique to neuropsychological journals: a "Dialog" section with topical debates between distinguished investigators such as theoretical approaches to memory; "Critical Reviews" and "Symposium" sections for empirical papers concentrating on single topics; and a "Rapid Communications" section containing mainly brief reports.

My guess is that neurologists will not be equally struck by *EJN's* uniqueness as a neurology journal. It is similar in organization and scope to *Neurology*, *Brain*, *Archives of Neurology* and other well-established publications. Its ability to provide rapid publication (three to six months) is a noteworthy feature, and the editors encourage paper submissions from investigators in Eastern Europe who might not otherwise publish in an English-language journal. This seemingly good journal, however, does not yet have a distinct identity.

Finally, although both *EJN* and *JINS* are similar in size, *JINS* is relatively inexpensive. Ultimately, the value of these two journals to institutions will depend on the discipline of the readers served. But it is my guess that at this stage *JINS* would be more sorely missed by neuropsychologists than *EJN* would be by neurologists. □

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Journals index

Adsorption	41	Analysis and Characterization	44
Alzheimer's Research	35	Invertebrate Neuroscience	34
Anaerobe	40	Journal of Biomedical Science	35
Aquatic Geochemistry	42	Journal of the International Neuropsychological Society	33
Auditory Neuroscience	34	Journal of Molecular Medicine	35
Autonomous Robots	43	Journal of Synchrotron Radiation	42
Bernoulli	40	Journal of Undergraduate Science	44
Biospectroscopy	37	Microbial Drug Resistance	36
The Chemical Intelligencer	41	Molecular Breeding	39
Complexity	43	Nature Medicine	35
Current Issues in Public Health	38	Neuroimmunomodulation	36
Current Opinion in Collid and Interface Science	41	The Neuroscientist	33
Cytokines and Molecular Therapy	36	Odyssey: The Glaxo Wellcome Journal of Innovation in Healthcare	38
Environment and History	40	Pharmaceutical News	38
European Journal of Neurology	33	RNA	37
Folding and Design	37	Science and Engineering Ethics	42
Genome Research	39	Science Spectra	38
International Journal of Forensic Document Examiners	44	Supramolecular Science	43
International Journal of Polymer			

Listening post

Christoph E. Schreiner

Auditory Neuroscience. Editor-in-chief Peter Dallos. *Harwood Academic*. 4/yr. ECU83, \$108 (personal).

THE increasing amount of important work in auditory neuroscience has led to the need for a new publishing outlet. *Auditory Neuroscience* is an attractive new addition to the sparse field of periodicals devoted to basic auditory research. According to Peter Dallos, the eminent editor-in-chief, not only is a journal needed to provide a forum for publication of important work, but it also has to be a first-class journal that promotes scientific excellence. It should have the highest editorial standards and be worthy of attracting the best papers in this sub-field of neuroscience. Judging from the sample issues, the editors are well on their way to achieving this goal. But it remains to be seen how well they can compete for top-quality work currently found in the main neuroscience journals.

This new journal attempts to cover all facets of auditory neuroscience, including cell and molecular biology; anatomy and physiology of the developing and the mature peripheral and central auditory system; behavioural and neuroethological aspects; and computational approaches. Each area has been assigned a prominent and capable associate editor.

About six or seven substantial, full-length papers appear in each issue, totalling around 16 printed pages. Short communications and reviews are also considered, although none has appeared so

far, and an informative editorial article prefaces the first two volumes. The reviews seem thorough and helpful, a fact apparently appreciated by many authors, who often acknowledge them in their papers.

The time between submission and acceptance is fairly short, averaging three-and-a-half months. The delay between acceptance and publication is harder to judge, as the issues do not indicate the month of publication, but it seems to be around three or four months. The layout, paper and figures, including half-tones, are all of high quality. There are no page charges or fees for colour plates, and the personal subscription rates are reasonable. The handling of the colour figures, though, is rather outdated and could be improved: they are assembled at the end of each issue rather than incorporated into the article.

Auditory Neuroscience follows in the best tradition of classical paper journals. Unfortunately, many libraries are having to reduce their subscription portfolios, which may impede the dissemination of this excellent material. In this age of widening computer networks and burgeoning electronic publishing, the journal might have a greater impact if, like many other leading neuroscience journals, it was computer-accessible. Nevertheless, it is already attracting consistently excellent research and is obligatory reading for anyone seriously interested in basic auditory research. □

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Neuro-specialist

Sten Grillner

Invertebrate Neuroscience. Editor-in-chief Peter N. R. Usherwood. *Sheffield Academic Press*. 4/yr. \$350, £245 (institutional); \$100, £70 (personal).

EACH species is of interest in its own right, whether mite, man or mollusc. Some, however, may also provide experimental preparations that reveal general biological principles common to all or part of the animal kingdom. Invertebrate neuroscience has provided several influential experimental models such as the squid giant axon and the crustacean stomatogastric ganglion. The main finding from these preparations went into the best journals available at the time. Of course, one hopes that this will always be the case. In neuroscience, there are now several excellent journals that primarily accept reports considered to be of broad significance. So why a journal specifically directed at members of only one large part of the animal kingdom with only one thing in common, the lack of a spine?

As a rule, studies in neuroscience provide new facts about a particular neural system or function. Most studies provide new findings but do not in themselves lead to new insights, and they may not even be particularly interesting. They are nevertheless needed to generate the factual base from which new bold hypotheses can be properly formulated. These 'fact-accumulating reports' often do not make it into the leading journals. So there may well be a place for a specialized journal for invertebrate neuroscience that can publish detailed high-quality reports.

Invertebrate Neuroscience has a very competent editorial board. Its format is similar to *Neuron*, with a few timely and brief reviews, followed by original papers. In each issue there is also a bibliography of the invertebrate papers published since the previous issue. So far, there have been several interesting original reports and well-written reviews.

The risk is that this attractive journal will receive due attention in the invertebrate neuroscience community but be overlooked by the rest of the neuroscience community. In the best of all possible worlds, one might instead wish for several specialized and problem-oriented journals, publishing both invertebrate and vertebrate papers on, for instance, learning and memory, development or motor control. In my view, such a scheme is more advantageous in the long run for both invertebrate and vertebrate neuroscience. □

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New Journals 1996

CRITERIA for journals to be considered for review in this issue were published in *Nature* earlier this year. They were that:

- (1) the first number appeared during or after June 1994 and at least four separate numbers were issued by the end of May 1996;
- (2) the journal is published at least three times a year;
- (3) the main language used is English; and
- (4) where possible at least four issues should be made available for review, including the first and the most recent numbers.

The time criteria ensure that a reasonable sample of issues is available for judgement by the time reviews are commissioned.

Several journals known to satisfy the criteria were not submitted for review or arrived too late for inclusion. It proved difficult to find reviewers for other, doubtless worthy journals, while some titles were considered to be of marginal interest to *Nature's* audience. Journals covering any aspect of science were eligible although those dealing with clinical medicine and pure mathematics were excluded, as were abstracts publications. A list of eligible titles submitted but not covered

appears below.

The brief given to the reviewers was to limit themselves to comments on the publications sent to them, and to avoid discussion of general questions of periodical publishing. As in previous years, the preponderance of journals in the biological sciences reflects the bias of the material submitted.

Details of editors and frequency of publication, and the subscription rates appearing at the top of each review, are given in most instances for 1996. This information is not complete in all cases, and readers interested in subscribing to a particular journal should check the rate with the publisher concerned. □

Journals also submitted for review

Archives of Suicide Research; *Chemistry — A European Journal*; *A Section of Angewandte Chemie*; *CVD: A Section of Advanced Materials*; *Current Opinion in Solid State and Materials Science*; *Environmental and Ecological Statistics*; *European Addiction Research*; *International Journal of Bio-Chromatography*; *International Journal of Rotating Machinery*; *Journal of Difference Equations and Applications*; *Journal of Dynamical and Control Systems*; *Journal of Inflammation*; *Journal of Neurovirology*; *Lifetime Data*; *Mental Retardation and Development Disabilities Research Reviews*; *Mineral Resources Engineering*; *Real-Time Imaging*; *Tissue Engineering*.

Dementia's demise

Mark P. Mattson

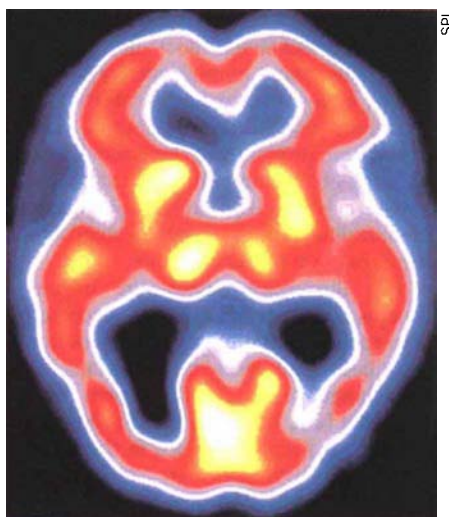
Alzheimer's Research. Editor-in-chief Sarah-Jane Richards. *Rapid Science*. 6/yr. USA \$340, elsewhere £200 (institutional); USA \$160, elsewhere £90 (personal).

AGE-RELATED neurological disorders are exacting increasingly heavy personal and financial tolls on society. Appropriately, governmental and private funding organizations are increasing their efforts to identify the causes of disorders such as Alzheimer's disease, and to develop preventive and therapeutic strategies for them. Journals devoted to a specific disease are certainly of great value in that they can focus and accelerate research efforts, an important goal of *Alzheimer's Research*. The editorial board consists of an excellent group of scientists whose collective expertise spans the field from molecular and cellular biology to clinical aspects of dementia. Because the key issue in the Alzheimer's field is identifying the cause(s) of nerve-cell degeneration, there is an appropriate weighting towards cellular and molecular aspects of neuronal degeneration.

Alzheimer's Research primarily publishes original research articles, with one or two short review articles per issue. A section called "Alzheimer's Focus" presents valuable brief synopses of recent high-profile articles and reports from recent meetings. Most articles published during the journal's first one-and-a-half years have addressed key issues in the field.

A large proportion of articles seem to involve independent repetitions of previously reported experiments. Although there is little glamour in confirming or contradicting earlier high-profile findings, reporting of such data provides an important avenue for consolidation of our understanding of the pathophysiological processes in Alzheimer's disease.

Articles are reviewed rapidly by contracted experts and published within 1–3 months of acceptance. There are clearly pros and cons of accelerated review and publication. On the positive side, access to recent data from other laboratories may allow researchers to accelerate their own efforts and so advance the field as a whole. On the other hand, if, through hasty review, spurious data are published, progress can actually be hindered. In the four sample issues, more than 30 per cent of the manuscripts were accepted for publication within one week of receipt, and roughly 80 per cent within one month. This clearly does not allow substantial revision of manuscripts and indicates that the articles may not contain the complete and thorough pieces of work that often arise



PET scan of an Alzheimer's-diseased brain.

following rigorous review.

Nevertheless, most of the articles published in the first issues seem to be of high quality and are timely, indicating that the editorial process is working and that this new journal is filling a void. The price of an institutional subscription is reasonable, but the personal rate is a bit high. □

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Happy reunion

Leonard I. Zon

Nature Medicine. Editor-in-chief Barbara J. Culliton. *Nature Publishing*. 12/yr. \$395, £350 (institutional); \$95, £90 (personal).

Journal of Molecular Medicine. Editor-in-chief D. Ganten. *Springer*. 12/yr. \$441, DM528 (institutional).

Journal of Biomedical Science. Editor-in-chief Chuan C. Chang. *Karger*. 6/yr. USA \$268.60, Europe SFr340.60, elsewhere SFr355 (institutional); USA \$43.80, Europe SFr52.80, elsewhere SFr60 (personal).

MUCH of modern biology has been spawned in response to a basic question of normal human physiology or the pathophysiology of disease states. Given this origin, it is reassuring that the number of applications of molecular biology to the practice of clinical medicine increases substantially every year. Despite this apparent success story, there is a growing schism between clinicians and basic scientists because of technological and linguistic barriers. One remedy is to provide a means for the two groups to converse better. To provide such a forum for the 'reunification'

of the two fields, several new journals have been created specifically to target molecular medicine.

The brightest new journal of this genre is *Nature Medicine*, as it generally follows the traditional style and rigour of its sister journal *Nature*. The layout is exciting, and flourishes with colour. "Commentaries" and "News and Views" are easily understood by clinicians and are detailed enough to please basic scientists. Book reviews appear in every issue. The editors have done a nice job maintaining the feel of a clinical journal. Most research articles appear within 3–4 months of submission. Several important articles published over the past year in *Nature Medicine* would traditionally have found their way into the *New England Journal of Medicine*. *Nature Medicine* will certainly be the principal journal for papers that bridge basic science and clinical medicine.

Journal of Molecular Medicine is a continuation of *Klinische Wochenschrift* ("The Clinical Investigator") originally published in 1864. As such, it maintains a rich tradition in blending basic research with clinical medicine. The journal particularly caters for regional submissions from Germany, but investigators from around the world do contribute. Its layout leaves room for improvement; the artwork is completely in black and white, and is occasionally underexposed. The original research articles are often short. The reviews are interesting and thorough, and are the highlight of the journal. The editors have had an excellent idea in starting a new column called "Clinical Implications", a forum for clinicians to reveal their insights into the marriage of science and medicine.

Journal of Biomedical Science similarly deals with molecular medical topics. Many submissions are from investigators in Taiwan. The artwork, reproductions and layout are done well. The journal offers rapid communication and publication; articles are often published two months after submission. Reviews are well written and timely with an emphasis on clinical-basic science issues. The original report section provides a forum for scientific correspondence; but the content of the correspondence seems only tangentially clinical, and often includes more cell biology and physiology than molecular biology.

It is no surprise that other journals such as *Molecular Medicine* have also appeared recently for communicating work that bridges clinical research and molecular biology. With the growing technology and advances in medicine and research, contributions to these new journals will ensure an exciting future for the union of basic science and medicine. □

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Brains, cytokines and nightmares

Carlos Martínez-A

Neuroimmunomodulation. Editors S. M. McCann and J. M. Lipton. Karger. 6/yr. USA \$514, elsewhere SFr668 (institutional); USA \$257, elsewhere SFr334 (personal).

WHAT is the meaning of a name? I guess that in searching for a title, the editors arrived at the answer Don Quixote gave to Sancho Panza: "it means what you understand of it". Thus *Neuroimmunomodulation* seeks to provide a home for new developments in a panoply of related disciplines covering the immunological, endocrinological and neurological sciences. The rapid increase in knowledge in these areas has been at the molecular level. But cellular and systemic perspectives are likely to be important in the future and might well become the principal areas of interest for this new journal.

Each issue aims to carry reviews as well as original articles. But the journal seems to be drifting towards becoming a reviews-oriented publication. As with many new periodicals, the quality of the papers is variable. Many of the contributors have direct links with members of the editorial board, which might explain the rapid manuscript acceptance speed. The journal is well produced, although the figures and tables look

slightly old-fashioned. Unfortunately, *Neuroimmunomodulation* does not yet allow authors to submit manuscripts by e-mail.

The issues reviewed concentrate mainly on cytokines and neuroendocrine control of inflammation. Given the limited success of many of the competing journals already in these areas, the future of *Neuroimmunomodulation* is not guaranteed unless it carves out a distinct identity.

But the more trendy the papers it accepts, the more likely it is to succeed. If it is to speak to immunologists, endocrinologists, neurobiologists and physicians in the same language and to break down the barriers that have long separated cellular- and molecular-oriented science in these fields, some improvements are still needed. Although the niche for such a journal might seem obvious, this is a challenging goal and I wish the editors the best of luck.

Let me end by quoting Cervantes again. In *Don Quixote de la Mancha*, Don Quixote tells Sancho Panza that it is a tradition among masters to promote their servants to governors in the kingdoms they conquer. But he points out that "the adventure of becoming governor of one of these kingdoms, although potentially of interest, is an adventure that in most of the cases ends in nightmare". I hope not on this occasion. □

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Superdrug or superbug?

Curtis Gemmell

Microbial Drug Resistance: Mechanisms, Epidemiology, and Disease. Editor Alexander Tomasz. Liebert. 4/yr. \$156.

THE threat that bacteria would defeat the best efforts of the pharmaceutical industry and become untreatable has been expounded over the years by many authors. It is no coincidence that the advent of this journal follows the appearance of multiple-drug-resistant *Mycobacterium tuberculosis*, especially among AIDS patients, and of epidemic methicillin-resistant *Staphylococcus aureus* as a hospital infection.

Medical microbiology in particular has witnessed a proliferation of specialist journals in recent years. Inevitably, there is a temporary withdrawal of support from well-established journals by authors attracted to the editorial board of a newcomer. Here board members contribute five out of eleven papers in the first issue, two out of thirteen in the third, and one out of nine in the fourth. Unfortunately,

the journal has had to resort to running the proceedings of a commercially sponsored symposium early in its history (the second issue). In the first issue, only five full papers and one brief case report constitute original previously unpublished findings; the third issue, though, contains a few more case reports as well as three reviews (two short and one long). Only by the fourth issue are there truly original papers, so there does seem to be a market, albeit fragile, for this journal.

There is also likely to be strong competition with two other well-established journals (*Antimicrobial Agents and Chemotherapy* and *Journal of Antimicrobial Chemotherapy*). It seems that many research groups are waiting in the wings to see whether the journal will establish its own niche. So my verdict at this stage (only possible in Scottish law) is "not proven". □

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Translation manual

Fran Balkwill

Cytokines and Molecular Therapy. Editors M. Talpaz and F. Herrmann. Dunitz. 4/yr. USA \$195, Europe £125, elsewhere £130 (institutional); USA \$99, elsewhere £65 (personal).

NATURE readers may think that 'translational research' has something to do with RNA and proteins, but a 'translational' scientist of the 1990s is more likely to be grappling with the tortuous transition of research from bench to bedside. An increasing interest in 'translating' advances in molecular biology to clinical problems such as cancer and autoimmunity has predictably led to a rash of new journals focusing on cytokine therapy, therapeutic immunology, gene therapy or a combination of all three. *Cytokines and Molecular Therapy* belongs to the latter category with its stated aim of "disseminating leading-edge research in the major fields of cytokines and molecular therapy".

Its main areas of interest are haematology, oncology and immunology, but articles from other specialities are encouraged. These interests are reflected in the contents of the sample issues sent for review. Each contains two or three detailed reviews and three to five original research papers. The reviews generally provide excellent, extensive and up-to-date perspectives of areas such as cytokine modulation of neonatal haemopoiesis, molecular approaches to cancer immunotherapy, the molecular biology of Gaucher disease, and the involvement of the interleukin-1 system in leukaemia. The quality and relevance of the original papers are rather more variable, as might be expected from a journal in its infancy. Some sort of editorial comment would also be useful, especially to place original articles in a wider context.

The editors and editorial board comprise a galaxy of the best clinical and translational scientists, which should guarantee the success of this journal. But there are strong competitors, both new and old. It remains to be seen how many good and original papers this demanding field of research will generate, papers ideally of the standard found in *Nature Medicine*. If there are not enough original papers to sustain all of these journals, *Cytokines and Molecular Therapy* would perform a worthwhile function by concentrating on its comprehensive and useful reviews. □

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Origami anyone?

Neville Kallenbach

Folding and Design. Editors Alan R. Fersht and Fred E. Cohen. *Current Biology*. 6/yr. \$459, £227.50 (institutional); \$159, £97.50 (personal).

THE subject of this journal, protein folding and design (with a bow to RNA as well), is among the most active and challenging areas of research in biophysics and biochemistry today. Research in this field covers a variety of theoretical and experimental approaches, from spectroscopy to genetics, with biomedical potential.

The editors say that articles dealing with the subject are now spread over a diverse array of journals, that the numbers of published papers with the words 'folding' and 'design' is on the increase and that referees with the necessary interdisciplinary skills are hard to identify. Although none of these claims alone is a compelling reason for launching a new publication, *Folding and Design* is by no means unwelcome.

How does it compare with major rivals in the field such as *Journal of Molecular Biology*, *Biochemistry* and *Proteins: Structure, Function and Genetics*, as well as newcomers such as *Protein Science* and *Nature*

Structural Biology? As with other Current Biology journals, this one has a particularly attractive format, with exceptional graphics. It offers a useful literature survey and is among the first wave of journals to go on-line. Editorial board members make up a distinguished international group, heavily slanted towards expertise in those proteins that reflect the editors' backgrounds.

The contents of the first three issues are variable in quality. Some of the short reviews are stimulating, whereas others are repetitive or pontifical. The research articles are uneven too, with important contributions next to inconclusive work.

Is there a niche for the journal? I am not aware of a shortage of space or lack of editorial enthusiasm for papers on folding in competing journals. But *Folding and Design* does seem to offer a centrality of focus that is missing in its rivals, apart from *Protein Science* and *Nature Structural Biology*. All three contain a high proportion of interesting titles, and offer attractive formats and rapid publication.

What gives one confidence that this journal will last is the organizational skill behind it. On the basis of the publisher's track record, I would give it good odds for survival. □

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Molecules for a living

Jacques R. Fresco

RNA. Editor Timothy W. Nilsen. Cambridge University Press. 12/yr. USA, Canada and Mexico \$360, elsewhere £288 (institutional); USA, Canada and Mexico \$180, elsewhere £168 (personal).

IF my research was still focused on the elucidation of the secondary and tertiary structures of ribonucleic acids, I would surely be a regular reader of *RNA*, a relatively new journal published on behalf of the RNA Society. Indeed, my continuing more general interest in the relationship between nucleic-acid structure and function and in molecular evolution requires that I peruse this well-formatted and well-illustrated journal fairly often.

As pointed out in the original statement of editorial policy, *RNA* touches on all aspects of ribonucleic-acid structure, biochemistry and molecular biology, relevant methodologies, structure-function relationships and theoretical considerations. Also as promised, the review procedure is rapid, as is subsequent publication of accepted papers, which, in general, appear to report high-quality and interesting research. Although very rapid publication and the promise not to intrude on

style may be a balm to the egos of researchers, they cannot be said to be achieved without some sacrifice of the elegance of the language of Shakespeare. Moreover, as original references appear sparse in *RNA* articles, this lack of journal intrusiveness also seems to go with the view that there is no need to make its readers aware that they "stand on the shoulders of giants".

This new journal fills a special need in allowing the presentation in one publication of subject matter that one formerly had to seek in many different sources. So that the journal might serve as a meeting of the minds of the RNA 'community', it was promised at the outset that *RNA* would publish perspectives, mini-reviews, book reviews, meeting reports and formal, more comprehensive reviews. The few efforts along these lines have so far been fairly successful, and one hopes for more of them.

If the role of a journal is to document and disseminate advances in a professional way, and to give coherence to a research area, *RNA* shows signs of succeeding. □

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Good for vibrations

Anthony Lee

Biospectroscopy. Editor Laurence. A. Nafie. Wiley. 6/yr. USA \$175, elsewhere \$205 (institutional); USA \$65, elsewhere \$95 (personal).

THE grass always looks greener on the other side of the fence. While biologists desperately claim to be interested in molecules, chemists are equally busy claiming that their studies of molecules are going to transform our understanding of 'life'. The result is many journals claiming to be interdisciplinary; some represent a true merging of approaches, others represent more a view by one side of what they think should be of significance to the other.

Biospectroscopy gives largely a chemist's view of what spectroscopy can tell us about biological molecules. An "editorial introduction" to the first issue describes the forms of spectroscopy to be included as "principally electronic absorption and fluorescence spectroscopy, optical activity, infrared and Raman vibrational spectroscopy, non-linear spectroscopy, time-resolved spectroscopy, electron spin resonance, EXAFS, and other related forms of spectroscopy". For a biochemist, the surprise in this list is the omission of nuclear magnetic resonance, but perhaps it is argued that such studies are reported in all the main biochemical journals, and journals such as *Journal of Magnetic Resonance* already cover more specialist, technical aspects of the technique. Results of other spectroscopic studies also, of course, appear in all the standard biochemical journals, and again more technical aspects are covered in several specialist journals as well as in journals such as *Analytical Biochemistry* and *Analytical Chemistry*.

The contents of the first volume of *Biospectroscopy* are heavily biased towards studies using infrared and Raman vibrational spectroscopies. Papers include studies on human breast tumours, children's teeth, calmodulin, angiotensin, exfoliated epithelial cells, and propranolol, showing the range of systems that can be studied in these ways. On the basis of the first issues, the papers are, however, much more likely to be of interest to actual practitioners of vibrational spectroscopy than to the general reader.

The journal is beautifully produced in a generous layout and on glossy paper. There is the occasional colour figure, a number of which, we are told, can be included free of charge. Subscription rates, both personal and institutional, seem very attractive. □

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Easy does it

Jon Turney

Science Spectra: The International Magazine of Contemporary Scientific Thought.

Editor-in-chief Gerhart Friedlander. *Gordon and Breach*. 4/yr. \$9.50, £5.95 (cover price).

Odyssey: The Glaxo Wellcome Journal of Innovation in Healthcare. Editor Andrea Dingemans. *PMSI Bugamor BV, PO Box 3008, 1300EK Almere, The Netherlands*. 3/yr. Free on application to Glaxo Wellcome or the publisher.

YOU are never going to read more than one or two of the academic journals reviewed here, of course. But you might want to add one of this pair to your browsing list as well, for those moments when you wonder what all those scientists who are misguided enough to work in other fields are up to.

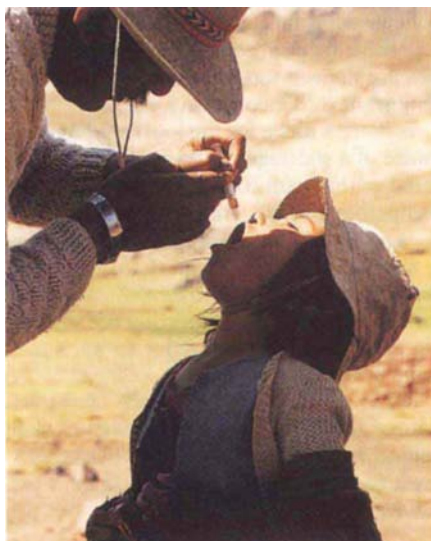
True, *Nature* will answer that question, in its way. But most of us welcome a more user-friendly presentation when we get out of field. *Science Spectra* is a new attempt to provide one, "a magazine for scientists, by scientists", according to the editor. The journal has several things going for it — the perennially worthy aim to foster communication between disciplines, lavish illustration and production, and an impressive roster of international contributors. What it does not have, in comparison with long-established contenders such as *Scientific American* or *New Scientist*, is a coherent editorial vision of the level and style of article to offer readers.

The result is a bag that is more mixed than it need be. The subjects are as various as you please, with eight or nine feature articles in each issue, although few would be novel to a regular reader of other science magazines. But, at the moment, the style varies too much for comfort. Issue one, for instance has a nice piece on why humans downgraded their sense of smell which assumes no knowledge whatsoever of evolutionary theory, but also carries a discussion of solar neutrinos that assumes that one knows what it means for a star to be on the main sequence, and can read a table of nuclear reactions. However, when the editors decide where to pitch their material, *Science Spectra* will be well worth a look.

Few such doubts with *Odyssey*. The magazine, written by a mixed group of journalists, scientists and doctors, focuses on latest developments in the life sciences, but only if they show clinical promise. There are also features on regulatory and policy issues, and well-chosen reflections on the research process. Although there are occasional editorial lapses in the review articles by scientists — an article on cytokines quickly degenerates into a list of molecular

biologists' acronyms — the contrast with the journalistic pieces is less marked than in *Science Spectra*. Perhaps the more unified focus on biomedicine helps to maintain coherence. Mainly rewarding reading, then, and yours for free. □

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From Odyssey

Polio vaccination in Bolivia.

Global welfare

George Gellert

Current Issues in Public Health. Editor Jonathan Mann. *Current Science*. 6/yr. \$129, £90 (institutional); \$65, £45 (personal); \$40, £30 (students/residents).

It can be difficult in such a multidisciplinary and fragmented field as public health to maintain an overview or distill practical knowledge. Even practitioners who studiously follow leading medical and public-health journals can find themselves overwhelmed or left behind on a vital and evolving public-health problem, controversy or innovation.

Hence *Current Issues in Public Health*. The journal contains 8–10 invited reviews by experts but written for a general audience of practitioners. It aims to fill a gap in continuing education by providing up-to-date information about important developments. Whereas other journals such as *American Journal of Public Health* present peer-reviewed results of original research, *Current Issues in Public Health* compiles, reviews and condenses this research along thematic lines and places it in a useful context for practitioners. Each issue contains four sections: public-health policy and practice; disciplines and themes; health of populations; and global health.

Has the journal achieved its objectives?

For the most part it has. The articles are concise and to the point, rarely exceeding four or five pages of text. But the reader might have valued the addition of editorials, letters or counterpoint views, as the invited reviews inevitably present only those issues that the (predominantly sole) authors see as meriting attention. The public-health issues surveyed are certainly important and timely, ranging from emerging and re-emerging infections to violence, under-served populations, environmental health and reform of the global health sector. The journal is a valuable applied resource in the literature for public-health practitioners. □

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Drug data

Anis Mir

Pharmaceutical News. Editor David J. Trigg. *Harwood Academic*. 6/yr. \$393 (institutional); \$252 (libraries); \$72 (personal).

WE are living in an era of momentous scientific and technological breakthroughs, changing disease epidemiologies and continual reforms in regulatory and healthcare economic policies. As a result of this pressure, the pharmaceutical industry is in a state of flux, reshaping its structures and strategy for drug discovery and development to cope with the rigours of change. For scientists working in pharmaceutical research and development, it is undoubtedly a formidable task to keep abreast of new developments and the emerging trends in such a varied and rapidly changing environment. It is perhaps not surprising that a new periodical appears that promises timely information on current developments in pharmaceutical science in all its aspects and with a global perspective.

In presentation and outlook, *Pharmaceutical News* reminds one of the prestigious *Trends* series of Elsevier. A close rival to *Pharmaceutical News* in style and content is the *Scrip Magazine: Pharmaceutical Issues in Perspective*, which with *Scrip Pharma Projects* and *Scrip World Pharmaceutical News* dominates the market for general pharmaceutical information.

The magazine will appeal broadly rather than to a specialized audience, with people associated with the pharmaceutical industry and pharmaceutical scientists in academic and medical institutions among its potential clientele. The contents consist of a regular leading article by the editor, David Trigg, that is generally topical and focused on health-care issues. Technical reviews and features on current topics are

informative and well written, and cover developments in pharmaceutical science and technology and clinical, regulatory, legal and health-care perspectives and issues. The general editorial policy is to commission articles, although many pieces are written by members of the advisory and contributing editorial boards, whose collective experience covers the length and breadth of know-how in the pharmaceutical sciences. Meeting reports, a witty news-and-views section and articles on case histories of drug design, discovery and awards are regular features. Each issue also boasts book reviews, new products and a meetings calendar.

The production is lavish, with a glossy format and abundant colour. An interesting initiative is that some issues carry a supplement entitled "Document Access Library", which consists of abstracts of papers to appear in forthcoming issues of Harwood Academic journals. Published bimonthly, *Pharmaceutical News* is good value for money by any measure; I for one wish it were published monthly. □

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Non-appliance of science

Robert Koebner

Molecular Breeding: New Strategies in Plant Improvement. Editor-in-chief Joseph N. M. Mol. *Kluwer*. 4/yr. \$239 (institutional); \$60 (personal).

PLANT molecular biology, long the poor relative of its animal and prokaryotic cousins, is enjoying a logarithmic growth phase, with no early end in sight as the main crop species have, one by one, become transformable. Commercialization of genetically engineered products has begun, and the stage is set for the exploitation of transgenesis to address many fundamental questions in plant biology.

And now we have *Molecular Breeding*, offering a publication time of 4–7 months and containing the conventional mix of full and short papers, meeting reports and mini-reviews. It expresses the high-sounding goal of seeking to "contribute to the understanding and progress of modern plant breeding, encompassing... biochemistry, genetics, physiology, pathology, plant breeding and ecology, among others". But superimposed on this is the clearly expressed over-arching focus on "plant molecular biology... leading to practical applications". I suspect that these aims are not entirely compatible, and this confusion will all too easily lead the journal away from one or other end of the spectrum

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

DNA gel analysis.

(and most probably, judging by the content so far, from the practical end). The problem with such a straying is that the journal would become a direct competitor with one of the well-established and well-patronized titles; in particular *Cell* and *Plant Molecular Biology* at the virgin pure end and *Theoretical and Applied Genetics* and *Molecular and General Genetics*, among others, at the only slightly soiled one.

A forward-looking reader might suggest that molecular techniques will increasingly find their way into plant-breeding practice, and that this journal therefore represents a conduit to facilitate the easy flow of these techniques from the academic laboratory to the plant breeder. The precedents for such a proposition are not good — *Theoretical and Applied Genetics* started life as *Der Züchter* ("The Breeder"), a change that has succeeded over the years in distancing it more and more from the 'practical' community of plant breeders. The result has been a widening gulf between what the breeders can do and do do, and what the 'biotechnologists' tell them they should be doing. I suspect that the appearance of the word 'molecular' in the journal's title betrays the reality that the editors are paying lip service to plant breeding; rather, their conscious or unconscious intention is to provide another stall in a crowded market for the airing of science that is still far from the applied. □

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Journal prices

Details of editors and frequency of publication, and the subscription rates given at the top of each review, are in most instances for 1996. Readers interested in a particular journal are therefore advised to check prices with the publisher.

Map reading

Peter Little

Genome Research. Editors A. Chakravarti, R. Gibbs, E. Green and R. Myers. Cold Spring Harbor Laboratory Press. 12/yr. USA \$495, elsewhere \$545 (institutional); USA \$95, elsewhere \$145 (personal); USA \$71.25, elsewhere \$121.25 (student).

GENOME projects can hardly be ignored, although some biologists may well wish they could. But what do we do with all the information? The obvious reply would be to put it in a computer and hope it makes sense to someone else. Remarkably, we still do not have truly archival databases of genetic data for many organisms, including humans, and this option is not really open. So enter *Genome Research*, a journal that grew out of *PCR: Methods and Applications* (for a review, see *Nature* 359, 447; 1991).

Research into the genome can be immensely dry — lists of clones, markers, maps and reagents — or immensely exciting — defining the basis of human disease, mapping the evolution of gene clusters against body plan, cloning genes that control body fat and so on. To many of us, genome analysis means both the dry and the exciting, because the maps, clones and markers are the tools of the exciting biology. *Genome Research* has positioned itself squarely into this view. The range of topics is quite extraordinarily diverse: biologically anonymous maps, positional cloning candidates, computational methods, genetic mapping results and strategies, reviews of post-genome-project biology, and technology of genetic analyses.

One constructive criticism: genome analysis is computer-intensive and I am slightly disappointed that there is no computerized data depository associated with the journal. It is crying out for one. Competitors? Of course: the most exciting results of genome research are published in many major journals but these do not cover the specialist or the small scale or the detail. The nearest competitor is *Genomics*, which is gradually moving towards the biological and genetic areas rather than genome-based approaches of *Genome Research*.

What this means is that the content of *Genome Research* will be essential to anyone involved practically in the field and most issues will contain information that could be used by any thoughtful molecular geneticist. To me, this is as good a definition of utility as I can produce: I wish the editors every success. □

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Bernoulli: Official Journal of the Bernoulli Society for Mathematical Statistics and Probability

Editor-in-chief O. E. Barndorff-Nielsen
Chapman and Hall. 4/yr. Europe £110, USA and Canada \$185, elsewhere £125 (institutional)

It is a bold step to claim the name *Bernoulli* for a journal limited to mathematical statistics and probability, for the members of this famous Basle family made many other important contributions to mathematics, including inventing the calculus of variations (in deriving the catenary), polar coordinates, the Bernoulli numbers of analysis (actually due to Johann Faulhaber in 1631, but then forgotten) and the Bernoulli equation of hydrodynamics. Yet there is some justification, for James (Jacob; 1654–1705) gave the first limit theorem in probability in his posthumous *Ars conjectandi*, Nicholas (Nikolaus; 1687–1759) substantially improved the limits of his uncle's theorem, and Daniel (1700–82) was one of several people who made early proposals of what is now known as the method of maximum likelihood. The new journal, moreover, is the official journal of the Bernoulli Society, founded in 1973 as a section of the International Statistical Institute.

The many existing journals devoted to probability and statistics will soon feel the effects of the newcomer, for *Bernoulli* has started strongly under its distinguished editors, and is exceptionally well printed on high-quality paper (oddly, the name of the printer is nowhere given). The early issues carry a promising mix of papers on stochastic processes and the mathematics of statistics, both areas that are developing strongly and which have important implications in many branches of science. There are no book reviews or (as yet) letters, but one or two papers are followed by discussions.

In an introductory note, D. G. Kendall, the first president of the Bernoulli Society, writes: "This is a Society that one confidently feels will live for ever". The same can be said of its journal.

A. W. F. Edwards Gonville and Caius College, Cambridge, CB2 1TA, UK.

Local attitudes

Euan G. Nisbet

Environment and History. Editor Richard H. Grove. *White Horse*. 3/yr. £60, DM150, \$100 (institutional); £30, DM75, \$50 (personal).

At Runnymede, west of London, is a small elegant memorial set up by American lawyers. Here King John, among other promises, pledged to relax his campaign to grow forests. The document he signed, Magna Carta, is not only the basis of human rights in anglophone nations: it also has much to say on environmental issues.

Environment and History is a potent if indigestible mix. Those who forget the errors of history are condemned to repeat them; those who forget the environment

risk becoming that history. As modern urban northerners justifiably campaign to save the tropical rainforests, we do well to remember past errors and successes.

Much of the emphasis in the first few issues is on forestry, especially in former British colonies in India, Africa and North America. This is appropriate, as much of our modern environmental concern about global vegetation grew out of early forestry. Then, as now, population was growing rapidly, in the imperial peace. The forest was under pressure. Conservation-minded foresters came into conflict with local entrepreneurs and aggressive peasants, often immigrants; older sustainable land-use practices were abandoned; governments vacillated.

The scientists made many mistakes and had much to learn, as *Environment and History* documents. Forestry as a science developed in Nancy, France, and in Germany. Indian forestry was dominated by three pioneer Germans: Brandis, Schlich and Ribbentrop. The problems they faced — fire management, for example — are discussed carefully and with some sympathy by the Indian contributors. The pioneers were extraordinary people. In Kipling's tale *In the Rukh* — the first of the Mowgli stories, later continued in the *Jungle Books* — appears a huge German, Müller, the Lord and Master of all the forests and woodlots of India. The terror of the government, he may be drawn from Brandis. The Mowgli stories epitomize the conservationist attitudes of the foresters.

African forestry had similar roots, drawn from Germany and Nancy, but with close ties to India. Older local people and foresters in former Zimbabwe (the subject of a special issue) tended towards conservationist attitudes while governments, peasant farmers and industry favoured exploitation. The debate continued until the Zimbabwe civil war and after.

The journal reflects this diversity of views. Many of the papers by local authors are impressively detailed, balanced and careful. Unfortunately, in some Western-authored papers there is also a large helping of sociobabble. And some papers are simply unusual. Is it really true that in the eastern mountains of Zimbabwe "contestations by Europeans and Africans become apparent in sexualisation of the landscape"? Perhaps, but I went to school there, and I did not see it. This is writers' country — for more profound insight it might be better to turn to Zimbabwe's authors, Doris Lessing, Dambudzo Marechera, Chenjerai Hove, who tread the same ground as the journal.

We need this journal, and we need it to be solid. Global change needs global policy, but living with environmental change needs local understanding. The lessons of the past century have much to teach us about managing biodiversity. Perhaps Kipling's German forester had it right: "I

tell you der big brass-hat pizness does not make der trees grow". □

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Airless life

John Postgate

Anaerobe. Editor-in-chief Larry Barton. *Academic*. 6/yr. £180, \$320 (institutional); £90, \$160 (personal).

ANAEROBES are microbes that flourish in the absence of oxygen. They are not a defined group because in fact microbes show a spectrum of oxygen sensitivities ranging from aerobes, which (like ourselves) have an absolute requirement for oxygen, to creatures killed by oxygen.

Judging from the spread of papers in the sample issues, bacteria are the dominant research subjects. Anaerobic micro-fungi appear a few times but anaerobic protozoa do not appear at all; facultative anaerobes — microbes capable of either aerobic or anaerobic growth — feature rarely, presumably depending on how far the research findings bear on anaerobiosis. The papers deal with clinical, taxonomic, eco-environment, biochemical, physiological, molecular genetical or even historical aspects of anaerobes. An off-beat item in the third issue is a letter giving a brief account of the anaerobic laboratory of the US National Institutes of Health, where a suitably garbed scientist can work anaerobically. Each issue includes a review article and there are occasional letters to the editor or summaries of scientific meetings.

One is always ambivalent about a new journal which, by tapping off papers from mainstream publications, increases the fragmentation of a discipline. But the fact that handling anaerobes is a tricky and specialized technology provides a certain justification for the present venture. The presentation is attractive; issues are slim but reasonable value for money; print, figures and layout are good; plates, on this showing, unimpressive. Despite some distinguished contributors, the quality of the papers is variable, most of them good but some rather pedestrian. But improvement can be foreseen: special learned societies for the study of anaerobes have been formed during the past decade in both the United States and the United Kingdom, as well as an International Society for Anaerobic Bacteria (*sic*), so there is every reason to expect that this journal, like its subjects, will fill an esoteric niche and flourish. □

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Candid chemistry

Harold Kroto

The Chemical Intelligencer. Editor-in-chief István Hargittai. Springer. 4/yr. \$69 (institutional); North America \$29, elsewhere \$33 (personal).

"INTELLIGENCER"? What does this word mean, I wondered. Perhaps the editor, István Hargittai, had actually got hold of the (in)famous Hungarian-English phrase-book (famous at least among Monty Python aficionados) that was originally used by John Cleese to buy matches — among other things. It turns out to mean 'bringer of information', in this case chemical (or chemistry?) information. But why, one might ask, should chemists, who already have a plethora of specialized publications catering to their professional interests, need yet another periodical?

The niche exists because chemists are a peculiar lot. For one thing, I know few who have not developed expertise in some other field, whether the arts, cooking, sport or what have you. I often wonder why so many became chemists when they could have been equally, if not more, successful pursuing their alternative passions — they would almost certainly have been richer.

Chemists also suffer from a curious professional form of schizophrenia: their thoughts are a mixture of abstract and hyper-realistic concepts at one and the same time; they are always subliminally aware that the intrinsic quantum properties of atoms and electrons govern all aspects of our everyday world in a way hidden from others. So the formula of benzene or its smell automatically triggers access to banks of information about the chemical and physical properties of this compound. Yet the general public may be only vaguely aware that benzene is an additive in petrol, if they have heard of the term at all.

There is one other important factor. It is the chip that rests on our communal shoulder: the fact that no-one has done more than chemists to improve the quality of everyday life and yet this contribution goes virtually unrecognized even though it daily stares everybody in the face. We chemists crave belated recognition of our modern Renaissance role.

Chemical Intelligencer covers this idiosyncratic range of interests. There are articles, interviews, notes, book reviews and letters on food, chemistry in Kuwait after the Iraqi invasion, the arts (writing, music, painting, sculpture), symmetry, postage stamps with chemistry themes, molecular models, the laboratory environment, historical articles, famous and infamous chemists (dead and alive), violins, photography — indeed anything that chemistry influences, which does not leave out much in the final analysis.

The quality of the articles is inevitably uneven, but the content is never dull. The magazine is well illustrated and includes a wealth of excellent photographs, diagrams and historically valuable archival material. As it makes its mark — as it undoubtedly soon will — it cannot fail to attract high-quality material. The unmistakable spirit of the editor, who has the charm to extract material — including frank interview revelations — from the busiest of scientists, pervades the pages.

There are as many different types of chemist as there are chemists, but I suspect that most would enjoy subscribing to this inexpensive, idiosyncratic but invariably fascinating publication. □

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Rising to the top

David A. King

Current Opinion in Colloid and Interface Science. Editors E. W. Kaler and B. H. Robinson. *Current Science*. 6/yr. \$612.50, £370.50 (institutional); \$212.50, £130.50 (personal); \$98.50, £63.50 (student).

Adsorption: Journal of the International Adsorption Society. Editor-in-chief K. S. Knaebel. *Kluwer*. 4/yr. Dfl456, \$319 (institutional).

It is a rare pleasure to be able to give an unqualified welcome to a new journal. I am delighted to be able to recommend research students and their senior colleagues working in colloid and interface science to subscribe to this new *Current Opinion* journal. The format has proved very successful in the biological and medical sciences, and there is clearly no reason why this should not also be the case in the physical sciences.

The formula is relatively simple. The whole subject matter covered by the journal is divided into 12 sections, and each section is to be covered once a year by short "subjective" reviews by experts working in the field. These reviews are usefully supplemented by bibliographies of relevant papers in the published literature, with the papers "of special interest" awarded one star (or bullet point) and those "of outstanding interest" two. I can think of no easier way of keeping abreast of the whole field than to browse through each issue, but the journal has archival value too. These reviews and the bibliographies will become indispensable to all future writers of reviews, chapters or monographs in colloid and interface science.

The true measure of the success of the

journal, and hence the editors, lies in the status of the reviewers who have been attracted for the first volumes. The list of section editors and their reviewers reads like a *Who's Who* of the field.

The topics have been well chosen, too. So far this year the following sections have been covered, each consisting of an overview by the section editors followed by between 8 and 12 subject reviews: scattering and surface forces; experimental self-assembly; imaging and other techniques; material aspects; theory of self-assembly; and thermodynamic and theoretical aspects. In the remaining three issues this year we are promised: rheology and rheological techniques; applications in chemistry and chemical engineering; surfactant science; colloidal aspects of biotechnology, biochemical engineering, pharmaceuticals and synthesis; dynamic aspects of colloids and interfaces; and food colloid emulsions, gels, foams and aerosols. The journal is well produced, in a good, easy-to-read format, and the editors, who must be working overtime to keep their deadlines, must be congratulated on the overall high standards they have established.

Adsorption is quite another matter. I have worked in the field of chemisorption for some 35 years, and so was surprised to discover that there is an established International Adsorption Society of which I knew nothing. This is the new house journal of the society, and, looking through the first few issues, I was unable to discover a distinctive flavour; is it trying to cover too much?

Most of the papers are from researchers in chemical engineering, dealing largely with the macroscopic properties of adsorption phenomena. This is clearly an important area. It bears little relationship to the detailed studies using spectroscopic and imaging techniques on well-defined single-crystal surfaces, with which I am more familiar, and this clearly explains my lack of familiarity with the society. A "Special Issue" does however cover molecular modelling of adsorption, indicating a wish to spread from applied to fundamental science. But there are good, well-established specialist journals that deal with these areas of adsorption studies, such as *Langmuir* and *Surface Science*. I am not convinced that the case is made for this addition.

I found the presentation irritating, even childish: oversized figures, many with the appearance of overheads prepared for seminars, with grossly oversized printing. Some good papers have been published in the first four issues, but I am not convinced that leading scientists in the field would send their best work here. □

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On the beam

Watson Fuller

Journal of Synchrotron Radiation. Editors S. S. Hasnain, J. R. Helliwell and H. Kamitsubo. *Munksgaard*. 6/yr. North America DKr2,845, elsewhere DKr2,750 (institutional); North America DKr595, elsewhere DKr500 (personal).

THIS is the most recent addition to the well-established and highly regarded journals and reference volumes published on behalf of the International Union of Crystallography. The editorial board reflects the now wide geographical distribution of synchrotron-radiation facilities and their users. Papers are invited on the whole range of synchrotron-radiation techniques and their application in biology, chemistry, materials science and physics. The emphasis is, however, intended to be on techniques rather than detailed analysis of the implications of the results for particular fields of study.

Although the journal has been going since October 1994, it is still fairly slim, each issue containing about half-a-dozen articles. But the quality of the articles is high, with a good representation of synchrotron sources and principal users from Western Europe, Russia, Japan and North America. The high-class presentation includes colour illustrations, offered free of charge.

Developments in the application of synchrotron research have benefited

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Close-up of polarized-proton generator.

enormously from fertilization between otherwise distinct research areas, and there is no doubt that this new journal will play an important part in furthering such interdisciplinary interactions.

All the articles published in 1996 appeared within seven months of receipt. This speed, together with the quality of the contributions so far and the high standard of production, makes the journal attractive to authors and required reading for workers in what worldwide is still a rapidly expanding field. □

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Flagship journal for a field at sea

William H. Casey

Aquatic Geochemistry. Editor-in-chief John W. Morse. *Kluwer*. 4/yr. \$253 (institutional); \$124 (personal).

THE dissipation of my field amuses me. More than a century ago, Jacobus Van't Hoff estimated the composition of Permian sea water by examining minerals in ancient evaporite beds. This effort failed, partly because of the high temperatures of rock formation, which he did not appreciate, and partly because a century of work would have been required to accumulate the necessary thermodynamic information about the important minerals and solutes. Now, however, geochemists, oceanographers, soil scientists, atmospheric chemists, environmental engineers and even the odd (aren't we all just a bit?) physical chemist can make reasonable predictions about minerals paragenesis and natural-water chemistry. The field has expanded well away from the Earth sciences, and this is enormously useful.

Aquatic Geochemistry reflects this very healthy expansion: John Morse, the editor-in-chief, has wisely established an editorial board by drawing on all of these lineages.

The articles range from the crustal cycling of elements on a geological timescale to the seasonal cycling of metals in lakes and to particular aspects of the toxicity of metals in water. The material is heavily biased towards inorganic processes, but this emphasis will change as new information is acquired about the distribution of soluble organic acids in nature.

The breadth of articles in *Aquatic Geochemistry* distinguishes it from the flagship journal in the field, *Geochimica Cosmochimica Acta*, and serves this protean community well. □

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Open channels

Brian Martin

Science and Engineering Ethics. Editors Stephanie J. Bird and Raymond Spier. *Opragen*, PO Box 54, Guildford, Surrey GU1 2YF, UK. 4/yr. \$130, £77 (institutional); \$66, £42 (personal).

DENIAL of authorship, misrepresentation in a *curriculum vitae*, misuse of grant money, denial of access to biological specimens, theft of patent rights — these are just some of the things I have been informed about over the past several months in long discussions with a few scientists and engineers. They provided plenty of documents too. In my studies of fraud and misrepresentation in science, it is apparent that the few publicized cases are the tip of an iceberg of ethical problems.

There is certainly plenty of material to be dealt with by *Science and Engineering Ethics*. Some topics receive a fair bit of attention, such as questions of authorship and data selection, with Millikan's oil-drop experiment the subject of much comment. But there are many others, including decision-making about genetic engineering, the hazards of whistleblowing, research on HIV infection, creative accounting and the right to die in dignity. Some important areas have not yet been treated, such as the ethics of weapons research. Imbalances are only to be expected; academic scientists and engineers, who have the greatest intellectual freedom, write most often about ethical issues that concern them personally.

As well as publishing papers — selected by double-blind refereeing, what else? — the journal includes articles about teaching ethics to scientists and engineers, reports on conferences and meetings and book reviews. One of its best features is the comments on papers, often giving strongly contrasting views.

The editors set out to get practising scientists and engineers to write about the ethical challenges they encounter on the job. A resulting weakness is an occasional lack of focus and unevenness in quality. More than compensating for this is the journal's accessibility and relevance. With contributors writing from a range of disciplines, often aimed at practical interventions to improve scientific practice, there are actually several articles that could usefully be given to colleagues or students. So the journal has kept open channels of communication and avoided becoming an in-group of professionals writing for each other. May it continue to do so. □

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Beyond the molecule

Harry L. Anderson

Supramolecular Science. Editor-in-chief Wolfgang Knoll. *Butterworth-Heinemann*. 6/yr. \$254, £170 (institutional).

SUPRAMOLECULAR chemistry proclaims that there is a chemistry beyond molecular chemistry: molecules can be programmed to organize themselves into supramolecular assemblies that are more than the sum of their parts. Over the past 20 years, the structures studied by supramolecular chemists have expanded into the realms of nanotechnology. It has become clear that understanding how to design small molecular components so that they self-assemble into functional supramolecular architectures is fundamental to both molecular biology and materials science; this area is highly interdisciplinary.

Supramolecular Science was launched to provide an interdisciplinary forum for papers in the emerging fields of nanotechnology, supramolecular assemblies and 'smart' materials. The editor-in-chief,

Wolfgang Knoll, identified a need to collect contributions previously published in many different journals with little overlap in readership. The journal is extremely broad in its scope. It aims to publish original peer-reviewed articles covering chemical design of functional materials, physical principles of self-assembly, biological approaches to intelligent materials, molecular engineering fabrication techniques, new techniques for structure determination at the molecular level and computational methods for predicting the properties of supramolecular aggregates. Each issue consists of about six original articles (short communications and full papers) and one "laboratory profile". These profiles are a refreshing change from the standard review format.

The journal has succeeded in attracting some high-quality papers, particularly in the areas of surface chemistry and colloids. But its broad scope means that it does not have a well-defined niche and I doubt whether it can compete with journals such as *Langmuir* and *Advanced Materials* which cover a similar area. □

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For freewheelers and wannabees

Ian Stewart

Complexity. Editor-in-chief Harold Morowitz. *Wiley*. 6/yr. USA \$195, elsewhere \$225 (institutional); USA \$69.95, elsewhere \$89.95 (personal); USA \$36, elsewhere \$66 (student).

I AM an unabashed fan of complexity theory, although not of the belief that it has all the answers, and I am far from convinced that its current methodology is anything other than a convenient stopgap. When we really start to understand how high-level structure emerges from low-level rules in complex systems, we will surely rely less on simulations and more on conceptual insights. But if we are ever to reach this happy state then the burgeoning activity in the area needs a focus. *Complexity* is the first and only specialist journal: how effective a focus is it?

Its academic pedigree is impeccable. Production quality is terrific: it looks like a newsstand magazine. Its contents are organized like a magazine too: editorial material, tutorial articles and surveys outweigh specialist papers. It is not a bad way to organize an interdisciplinary journal. Specialist articles ought to be able to survive in journals appropriate to their own applied area, and will be far more widely read there than in a ghetto publication — but where can you read or publish free-

wheeling discussions of broader issues?

The variety of contributions is wonderful: linguistic patterns, genetic algorithms, financial markets, technological evolution, phase transitions as universal principles, artificial intelligence, cellular automata, order and disorder in ant colonies. The first few issues are perhaps a bit too self-conscious, but it is not easy to start up something of this kind without some backward glances. This is a house journal for complexity theorists and wannabees, with plenty to offer any interested scientist or mathematician who has the inclination and imagination to ask the hard questions.

As Harold Morowitz says in a perceptive opening editorial, the hierarchical organization of science has given us a deep understanding of many levels of the Universe, from atoms to ecosystems, but what it has not given us is any comparable understanding of the links from one level to the next. The world is full of scientists who confidently assert that these links exist, but few can offer any serious insights into what is actually going on. If you think such questions are worthy of serious effort, *Complexity* is for you. □

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Independence play

Dave Cliff

Autonomous Robots. Editor George A. Bekey. *Kluwer*. 4/yr. DFI468, \$268 (institutional); DFI250, \$105 (personal).

ALTHOUGH malignant androids with human-level intelligence are likely always to be fiction, they have one property that it is both desirable and realistic to attempt to build into future models of industrial or commercial robots: autonomy. Like familiar industrial robots, an autonomous robot has sensors (for example, video cameras) and actuators (for example, motors); but,

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Getting to grips with robotics.

crucially, it is responsible for achieving desired tasks by coordinating its own perceptions and actions for extended periods of time without human intervention. Autonomous robots are typically mobile and self-sufficient. The ability to function independently, reacting appropriately to unpredictable or changing environments, opens up several important areas of application and presents many challenging research issues.

Unlike most established robotics journals, this new one is devoted solely to autonomous robots. Its stated aims and scope include robots that display mobility on land, under water, in the air or in space; the mobility may be provided by wheels, legs, fins, rotors or other propulsion devices. Such mobility requires research in sensing, in computer learning, control and adaptation, and in self-calibration and self-repair. There is only one journal I know of that has a similar emphasis on autonomy, *Robotics and Autonomous Systems*.

Whether there is sufficient interest to sustain two journals remains to be seen. Certainly *Autonomous Robots* has several strong points in its favour. The 30-member international editorial board includes many of the people who helped to shape the

study of autonomous robots into a recognizable discipline. In addition to technical papers reporting theoretical advances and application examples, the journal accepts papers describing prototype systems as well as tutorials, surveys and essays offering distinct perspectives. There has also been a special issue on autonomous robots for planetary exploration. All the papers seem to be of a high standard expected. The typography and production standards are also good. Good value for money, it deserves to succeed. □

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Young pretender

Laura Garwin

Journal of Undergraduate Sciences. Editors V. Jain *et al.* Variable frequency. For further details contact the subscription department at PO Box 380932, Cambridge, Massachusetts 02238, USA.

GET a few editors of science journals together over a few beers, and it won't be long before the conversation turns to the use of published papers as 'performance indicators', and the distortions that this practice introduces into the publication process. Whereas we editors still cherish the idea that papers are written to convey new research results to fellow scientists who can make some use of them, there are others out there for whom the specific content of published papers seems to matter less than their mere existence.

So I find it rather disturbing that Harvard University's *Journal of Undergraduate Sciences* — created in 1994 to publish the results of undergraduate research projects — claims in its inaugural issue to be a place for "graduate schools... science award administrators, and professors... to discover promising young scientists". While most journals reconcile themselves to being used in this way, *JUS* seems to welcome it with open arms.

Of course, *JUS* sees itself as more than a marketing device for Harvard undergraduates; its main stated aims are to foster interdisciplinary communication and to encourage undergraduate scientists to write clearly and concisely. The journal is indeed wide-ranging — covering all the sciences, together with history of science and public policy — but then so are other general journals, such as *Nature* and *Science*. A *JUS* editorial complains that papers in these journals are "too focused and too technical" for the undergraduate reader (probably a fair accusation), but then the abstract of a paper in the same issue starts with a sentence ("The *Drosophila*

International Journal of Polymer Analysis and Characterization

Editor-in-chief H. G. Barth
Gordon and Breach. 4/yr. £340
(institutional); £218 (library); £75 (personal).

WITH the continuing development of new polymers that may not be sensitive to well-developed characterization methods, new reliable approaches must be devised to evaluate performance. But, as the editors point out, papers detailing such advances are scattered throughout the many existing polymer journals.

A single forum for such articles will, they claim, make it easier for researchers to find this information. Yet despite this emphasis, some of the papers, like those in the older journals, use the results of analysis or characterization to explore theoretical predictions or define physico-chemical behaviour. Not surprisingly, over a third are on chromatographic analysis.

Each issue contains around seven papers (short, long; 'how to', 'what if?'). The printing is clear and the layout good, although figures seem to be reproduced as supplied by the authors. Publication rate is slow (nearly two years between submission and appearance).

But does the journal really fulfil a need? Articles can now be tracked down easily and cheaply using sophisticated software and electronic databases. The journal certainly has a place in industrial and academic establishments where characterization and analysis are important. But many of the papers are of sufficiently good quality to make it into established polymer journals, so its success will depend on how well it can compete.

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International Journal of Forensic Document Examiners

Editor Joel S. Harris
Shunderson Communications, PO Box 42057, Ottawa, Ontario, Canada K1K 4L8.
\$40 (institutions); USA and Canada, \$30, elsewhere \$35 (personal)

IN 1974 the American Academy of Forensic Sciences established a certification programme for several fields, including organization of an American Board of Forensic Document Examiners. No academic institution yet awards a degree in forensic document examination, but there are now around 200 certified diplomates in the US and Canada.

The need for examination of documents arises mainly in criminal cases. *International Journal of Forensic Document Examiners* claims to be the first journal devoted entirely to this subject. Its presents peer-reviewed research articles, technical reports and case studies on subjects including handwriting, mechanical printing devices, security documents, plastic cards, altered documents, charred and/or water-soaked documents and chemical analysis of media used in document preparation.

Nearly all of the contents consist of reprints from other sources. Most articles were presented at conferences. The journal also publishes abstracts of papers and accounts of news based on reports in newspapers, and offers to print free announcements of relevant meetings. Descriptions of new equipment appear, but the distinction between editorial reports and paid advertisements is not made clear. Most reports are anecdotal and there is little of general scientific interest. But the journal serves a small group of specialized technologists and presents an abundance of material.

Lee Loevinger Hogan and Hartson, 555 Thirteenth Street, NW, Washington DC 20004-1109, USA.

melanogaster gene *corkscrew* (*csw*) encodes a tyrosine phosphatase important for transducing the signal from a number of receptor tyrosine kinases (RTK's), including *torso*, *sevenless*, the fly EGF-receptor homolog *torpedo*, and the fly FGF-receptor homolog *breathless*...) guaranteed to scare away the most resolute physics undergraduate — or PhD, for that matter.

Sadly, the guide to authors positively encourages bad habits, such as the use of the passive voice (which can actively hinder comprehension, by obscuring the distinction between new work and old) and the confusing use of the plural pronoun 'we' for a single author. Even more worrying is the suggestion that "if your original hypothesis was not validated by your results, then it is appropriate to analyze your methods and procedures for systematic errors or incomplete control of all variables" — surely it is appropriate to do this whether or not one's hypothesis has been validated!

The journal is proud of its peer-review process, undertaken by Harvard graduate students and professors. But how rigorous can such a process be, when other journals have to comb several continents for truly

expert referees? The answer is that, for all its worthy aims, *JUS* is really just a make-believe journal. The papers fall into two categories: those that are 'just' undergraduate research papers (some have reference lists that cite only textbooks, with perhaps a sprinkling of very old research papers) and those that may be valuable contributions to the scientific literature. The former don't need to be published at all — which isn't to say that they shouldn't be written, and examined critically by professors — but the latter ought to be published in a 'real' journal, where they will be seen by others in the field. *JUS* seems to care about everything except what a journal is really for — the communication of research results to the relevant audience.

Undergraduate scientists do need to learn how to write clearly and concisely, but also how to judge whether their results make a valuable new contribution to a field of knowledge. Allowing them to be big fish in a small pond — even a highly regarded pond — is likely to stunt their growth. □

Laura Garwin is physical sciences editor of *Nature*.

Sub-galactic clumps at a redshift of 2.39 and implications for galaxy formation

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A large number of very faint, compact objects have been found at a redshift of 2.39 in optical images of the distant Universe. The objects appear to be star-forming spheroids smaller than the bulge of a spiral galaxy; they are much smaller and fainter than typical galaxies seen today. These objects may be part of a reservoir from which many of today's luminous galaxies were formed through repeated mergers.

EXPLAINING the formation of the luminous elliptical and spiral galaxies is one of the most challenging problems in observational cosmology. Put in the most general terms, galaxies form by the condensation of matter that was once much more uniformly distributed in the Universe. Astronomers have been working for decades to advance our knowledge in this area. Increasingly sophisticated numerical simulations of the early Universe take into account effects of the so-called 'dark matter', matter that is believed to dominate the Universe gravitationally, but which emits no radiation. Utilizing various types of dark matter ('hot', 'cold' or 'mixed') in combination with normal (baryonic) matter, these models have substantially improved our original simple picture of galaxy formation from large rotating and collapsing gas clouds, or 'protogalaxies'. Recent advances from several deep-redshift surveys and the results from the COBE mission, which measured temperature fluctuations in the microwave background ($\Delta T/T$) of about 10^{-5} at a redshift of 1,000, have placed limits on when galaxies may have begun to form and when they probably stopped forming. There are several excellent discussions of galaxy formation¹⁻⁴.

Among the many theories of formation, the idea that galaxies may form by the accumulation of smaller star-forming subsystems has recently received much attention⁵⁻⁸. Two major lines of evidence lend support to this 'bottom-up' scheme. First, deep Hubble Space Telescope (HST) images and the size-redshift (θ - z) relation of luminous early-type galaxies (E/S0) and mid-type spiral galaxies (Sabc) indicate that these objects were assembled largely before $z \gtrsim 1$ and have been evolving passively since $z \lesssim 1$ (refs 9, 10). In addition, there is evidence from both HST and ground-based work that the galaxy-merger rate was higher in the past, roughly increasing with redshift in proportion to $(1+z)^m$, with $m \approx 2-3$ (refs 11-13). Taken together, these two points suggest that the luminous galaxies we see today could have been assembled from the merging of smaller systems sometime before $z \gtrsim 1$. The second piece of evidence comes from the excess of blue objects at faint magnitudes which has been found in many deep imaging studies, for example, ref. 14. Although recent spectroscopic studies¹⁵⁻¹⁷ have found many of these faint blue galaxies (FBGs) to be at modest redshifts ($z \approx 0.5$), the existence of a significant, high-redshift 'tail' to the distribution^{18,19} raises the possibility that a non-negligible fraction lies at $z \gtrsim 2$. It has been noted²⁰ that for $B \gtrsim 25$ mag, the FBGs are extremely compact ($\leq 0.2-0.3$ arcsec). This likely higher-redshift FBG population, which is dominated by late-type or irregular galaxies⁹ that have undergone substantial evolution since $z \lesssim 1$, could provide a reservoir of building blocks from which the luminous, present-day galaxies were made at $z \gtrsim 1$. Indeed, to produce the number of giant galaxies seen today, many more objects at $z \gtrsim 2$ are

required by most merger-dependent models of galaxy evolution^{18,21} than we see for $z \lesssim 1$. Recent findings, such as a population of faint ($B \gtrsim 24$ mag) blue galaxies at $1 \lesssim z \lesssim 3.5$ with rather unusual morphologies which suggest dynamical formation processes or mergers^{7,8,18}, seem to support this merging hypothesis.

To resolve this issue, direct observational evidence is critical. The difficulty is in finding large numbers of faint, and presumably distant, galaxies at or near their time of formation. There exist only a few known examples of galaxy clusters or groups with spectroscopic confirmation at very high redshift^{7,22-25}. Many theories of galaxy formation predict that there should be a widespread population of primeval galaxies²⁶ at a cosmic epoch somewhere before $z \gtrsim 2$. These primeval galaxies, undergoing an intense initial burst of star formation, should be detectable in several atomic emission lines which arise from the resulting hot ionized gases (for example Lyman- α ; ref. 27). Exhaustive ground-based, Lyman- α searches for such young star-forming objects over the past 15 years^{28,29} have been remarkably unsuccessful for several possible reasons: first, even small amounts of dust, if properly distributed, can effectively quench Lyman- α emission³⁰; second, the objects may primarily occur at much higher redshifts ($z > 5-10$) than have thus far been explored; third, they might exist in protoclusters or large-scale structures at high redshifts (although the clustering amplitude is predicted to be lower at high redshifts, by cold-dark-matter theories), causing most narrow-band searches to look in between any such structures; fourth, the objects may lie beyond our ground-based flux detection limits. Finding such objects, or a similar population of very compact star-forming systems, may help us to improve our understanding of how galaxies are formed.

Here we report evidence in support of the 'bottom-up' hypothesis, in the form of the spectroscopic detection of Lyman- α emission from five subgalactic-sized clumps at $z \approx 2.39$, and the possible detection of such emission from 13 other, similar objects at the same redshift in deep HST images. These objects are labelled in Fig. 1, which covers about 2.5×2.5 arcmin of the sky or $\sim 0.7 \times 0.7$ Mpc at $z \approx 2.39$ ($H_0 = 80 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and deceleration parameter $q_0 = 0.5$ throughout), and may be the building blocks from which present-day galaxies were made. These $z \approx 2.39$ candidates could be evidence that galaxies, or the star-forming subsystems from which they formed, tended to exist in groups or protoclusters in the early Universe.

A rich group of objects at $z \approx 2.39$

The initial discovery of a possible group at $z \approx 2.39$ was made from ground-based photometry with a medium-band (150 Å-wide) filter centred at 4,130 Å (Lyman- α at $z \approx 2.39$) in the field surrounding the weak radio galaxy 53W002 at $z = 2.390$ (ref. 31),

yielding two other candidates at the same redshift⁷. These were later spectroscopically confirmed with the Multiple Mirror Telescope (MMT), as shown in Fig. 2. Fortunately, the existence of a nearly identical, medium-band filter on the HST (F410M, centred at 4,100 Å) allowed the same observations to be conducted with higher sensitivity at a much higher spatial resolution than could be achieved from the ground. The HST is able to achieve nearly the full point-source sensitivity gain which is lacking in ground-based data, because of the characteristically small sizes of the $z \approx 2.39$ candidates.

The new HST data are presented in the colour–colour diagram in Fig. 3, which shows photometry for 115 objects detected simultaneously in the broad-band Wide Field Planetary Camera 2 (WFPC2) filters F606W (V) and F450W (B), and in the medium-band filter F410M (Lyman- α at $z \approx 2.39$). As with the ground-based data, the Lyman- α passband is contained entirely inside the B_{F450W} filter, so that proper continuum subtraction is possible. At $z \approx 2.39$, these bands sample the emitted wavelengths of 1,200–2,100 Å, where the spectra of star-forming galaxies are relatively flat³¹.

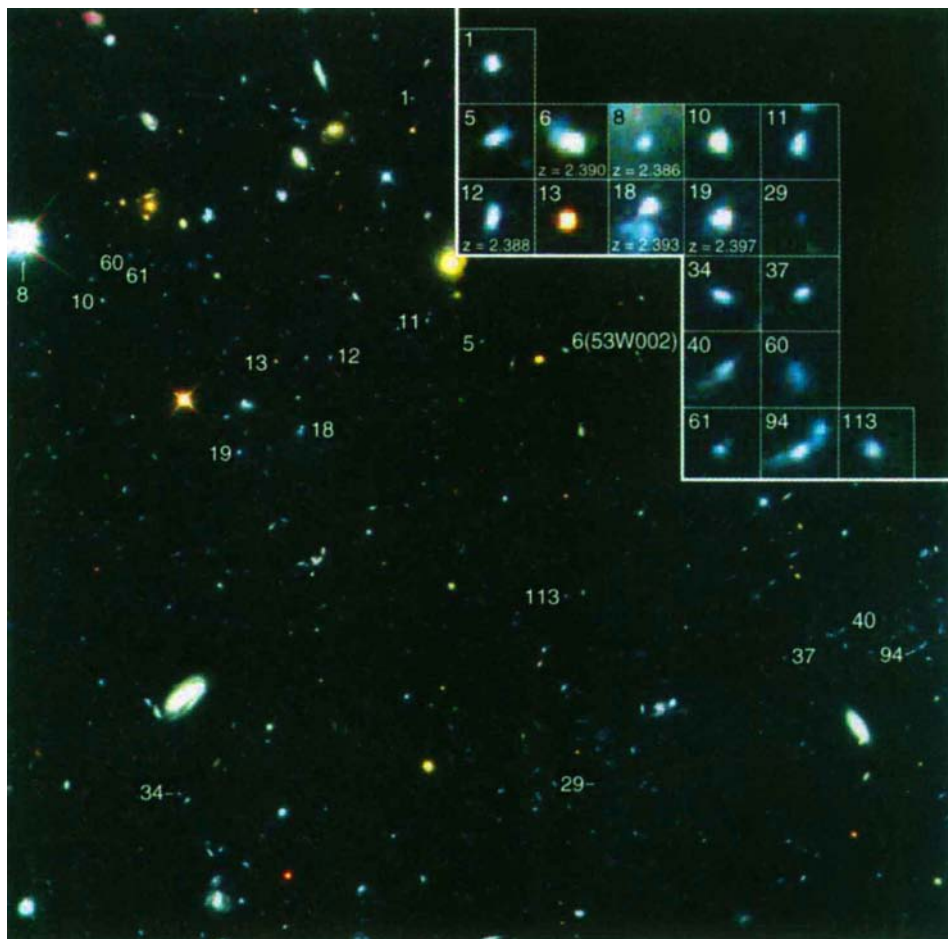
The solid line in Fig. 3 shows the expected relationship for objects with a flat, power-law spectrum having frequency-dependent flux F_ν proportional to $\nu^{-\alpha}$, where ν is frequency and α is the power-law spectral index ($F_\nu \propto \nu^{-\alpha}$) across these three adjacent filters; indeed most of the general field objects are distributed around it. There is a group of 18 objects which are at least two standard deviations below this line, showing that they have significant emission in F410M, which is probably Lyman- α emission at $z \approx 2.39$. Five of these are spectroscopically confirmed (Fig. 2), out of seven objects for which spectra were obtained, indicating this method of finding compact $z \approx 2.39$ candidates is

over 70% reliable. Apart from the three brighter objects, which were easily seen in ground-based photometry⁷ and contained weak active galactic nuclei (AGN), most of the sample has Lyman- α emission with average rest frame equivalent widths (estimated from the F410M photometry) of only 40–50 Å—more typical of ionization arising purely from star formation²⁷. This is in excellent agreement with recent findings of a population of normal (no AGN), star-forming objects at $3.0 \lesssim z \lesssim 3.25$, whose spectra show both stellar and interstellar absorption lines and Lyman- α emission with equivalent widths $\lesssim 20$ Å (ref. 8).

Although some of these candidates could be foreground galaxies with O II] (at 3,727 Å) redshifted into the F410M filter (at $z \approx 0.097$), such intrinsically faint and compact objects would be quite unusual at such a low redshift. No other strong emission lines exist for star-forming objects between 1,216 and 3,727 Å and the differential volume element at $z \approx 2.39$ is ~ 20 times larger than at $z \approx 0.097$, implying that most of the 18 significant candidates are probably at $z \approx 2.39$.

The reliability of our eighteen $z \approx 2.39$ candidates (Fig. 3) is strengthened further by the fact that three of the spectroscopically confirmed members are at the lower boundary in the $m_{F410M} - B_{F450W}$ colour of the general field population. One of the candidates which has been rejected by spectroscopy was the reddest object below the power-law locus, and has a spectrum similar to that of a faint M star. The other red candidates may also be M stars as the spectrum of an M star can give large negative $m_{F410M} - B_{F450W}$ indices. It is of course possible that there exist additional members of the group, which are not in Fig. 3 because they are obscured by dust which has quenched the Lyman- α emission by resonant scattering. According to the galaxy counts in this field, however, there cannot be more than about 60

FIG. 1 True-colour image of a combined 48-orbit HST WFPC2 exposure in B_{F450W} (blue, 16 hours), V_{F606W} (green, 5.7 hours) and I_{F814W} (red, 5.7 hours). The image covers about 2.5×2.5 arcmin ($\sim 0.7 \times 0.7$ Mpc at $z \approx 2.39$), and the V_{F606W} image has been rotated by -6.7° to match the B_{F450W} and I_{F814W} image orientations, resulting in slanted borders. Labelled objects are $z \approx 2.39$ candidates with significant excess emission in the WFPC2 F410M filter (see Fig. 3). The insets in the upper right of the image are enlargements of the 18 candidates, covering about 1.8×1.8 arcsec each. The sky-background in the image is $\sim 1-3$ mag darker than from the ground (23.94 mag arcsec⁻² in B_{F450W} , 23.01 in V_{F606W} and 22.45 in I_{F814W}). The resulting 5σ point source sensitivity limits are $B_{F450W} \lesssim 28.4$ mag and $I_{F814W} \lesssim 27.6$ mag, and the 1σ surface brightness sensitivity limits are $B_{F450W} \lesssim 27.5$ and $I_{F814W} \lesssim 26$ mag arcsec⁻². In comparison to the FBG population as studied by, for example, Glazebrook et al.¹⁶, which for $22.5 < B < 24$ has mean $B - V \approx 0.75$ mag (as estimated from their $B - R$ colours), our candidates have an average $B_{F450W} - V_{F606W} \approx 0.6$ mag. The $z \approx 2.39$ candidates have an approximate space density of 0.027 Mpc^{-3} ($q_0 = 0.5$) and 0.012 Mpc^{-3} ($q_0 = 0$). In comparison, Driver et al.⁹ find a space density of galaxies with comparable luminosities of only $\sim 0.01 \text{ Mpc}^{-3}$ locally ($q_0 = 0.5$). We do not believe that the discovery of our $z \approx 2.39$ group is strongly biased by targeting the weak radio galaxy 53W002 because the galaxy counts in this field are consistent with those in several other randomly selected fields^{50,51}, and the surface density of such weak radio sources is high enough that one would be found in every few WFPC2 fields⁵².



additional objects without producing a significant excess in the broad-band counts, which was not observed²⁰. The actual number of undiscovered $z \approx 2.39$ objects is probably much less than this, of the order of 20–30, or else more ‘spikes’ in the redshift distribution would have been seen by now at $z > 1$ in deep-redshift surveys elsewhere.

Faint blue sub-galactic clumps

Sizes. Figure 4a shows the size distribution for the $z \approx 2.39$ candidates and indicates that most are very compact, with half-light radii, $r_{\text{HL}} \lesssim 0.1\text{--}0.2$ arcsec, or $0.5\text{--}1.0$ kpc. The objects, whose cores are in many cases barely resolved by the HST, are typically detected at least 3–4 scale lengths (or average radii) out, indicating that the scale length measurements are robust in these deep images (see Fig. 5, which also shows a typical stellar profile for comparison). All candidates are much smaller than the median scale length of WFPC2 field galaxies at the same flux levels, which is about $0.3\text{--}0.4$ arcsec (ref. 20).

Average light profiles were generated for the candidates, assuming that they are all to first order similar in shape and size. These profiles are shown in Fig. 5 and are, for each filter, better fitted by an $r^{1/4}$ bulge-like profile (where r is the elliptically averaged radius) than by a disk-like exponential. The continuum scale lengths of the candidates have a mean value of 0.11 arcsec (≈ 0.5 kpc at $z \approx 2.39$) and are very similar in B_{F450W} , V_{F606W} and I_{F814W} , showing no dependency on rest-frame wavelength below $\sim 4,000$ Å.

The question arises as to whether or not we are seeing the full extent of these objects. The K -correction (the difference in an object’s spectral-energy distribution between the rest and observed wavelengths due to its redshift) for young spectral-energy distributions at $z \approx 2.39$ could have compensated for at least some of the cosmological $(1+z)^4$ surface brightness dimming³¹. Unlike the initial burst of star formation that may take place in ellipticals and the bulges of spirals, disk star formation rates can be almost constant with time. Disks can, therefore, actually brighten towards decreasing redshift for a significant time, eventually reaching an approximately steady level until gas exhaustion. However, with the exception of 53W002 itself, which already has at $z \approx 2.39$ a well-developed $r^{1/4}$ -law profile with a large scale length indicating a massive early-type galaxy³² (and was by selection included in this WFPC2 field), it appears that none of the other candidates are yet fully assembled massive ellipticals or spirals. Their scale lengths are quite comparable to those of bulges in local spirals³³, which range from 0.2 to 4 kpc with a type-dependent median that is close to 1 kpc for types S0–Sbc. Given that the average value of the bulge-to-disk scale length ratio of nearby late-type galaxies is $\sim 0.07 \pm 0.04$ (ref. 34), these $z \approx 2.39$ candidates may be sub-galactic-sized (compact) and young (blue) spheroids, possibly representing the bulges of young galaxies that have not (yet) developed significant disks around them, and/or disks that are reduced in brightness in the HST images by the severe cosmological surface-brightness dimming.

Luminosities. The implied luminosities at $z \approx 2.39$ for all candidates are shown in Fig. 4b, and typically range from $M_V \approx -23$ to -18 mag (based on the stellar population models, age estimates and K -corrections as described in ref. 31). Subtraction of any contributions from AGN was done following the point-source subtraction method of ref. 32. The (evolving) absolute magnitude of an L^* galaxy (a galaxy of luminosity $\sim 10^{11} L_\odot$) at $z \approx 2.39$ is $M_V \approx -23$ mag, and was estimated by assuming that there would have been ~ 2 mag of stellar evolution since $z \approx 2.39$ for a typical starburst $\sim (0.3\text{--}0.5) \times 10^9$ years earlier (as their unreddened blue colours suggest; Fig. 3)^{31,35}. The adopted ~ 2 mag of luminosity evolution has an uncertainty of $\sim 0.50\text{--}0.75$ mag and comes from the models used in ref. 31 for their $z \approx 2.4$ object. Therefore, if indeed their stellar populations are young, most of our candidates have luminosities of $\lesssim 0.1\text{--}1 L^*$, and so possibly had only $\sim 10^9\text{--}10^{10} M_\odot$ processed into stars at $z \approx 2.39$. For these parameters, the free-fall time expected for these clumps is $\sim (2\text{--}4) \times 10^8$ years, long

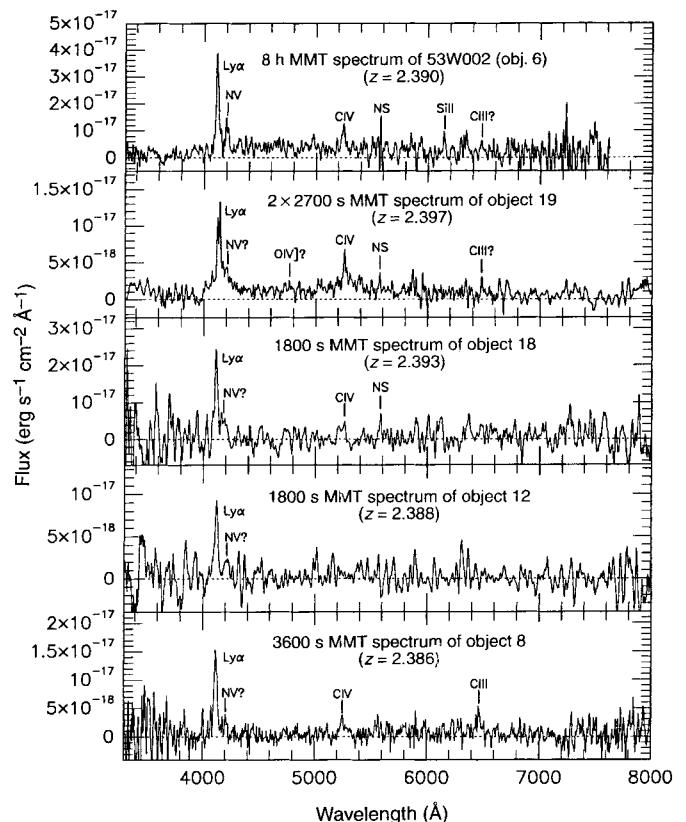


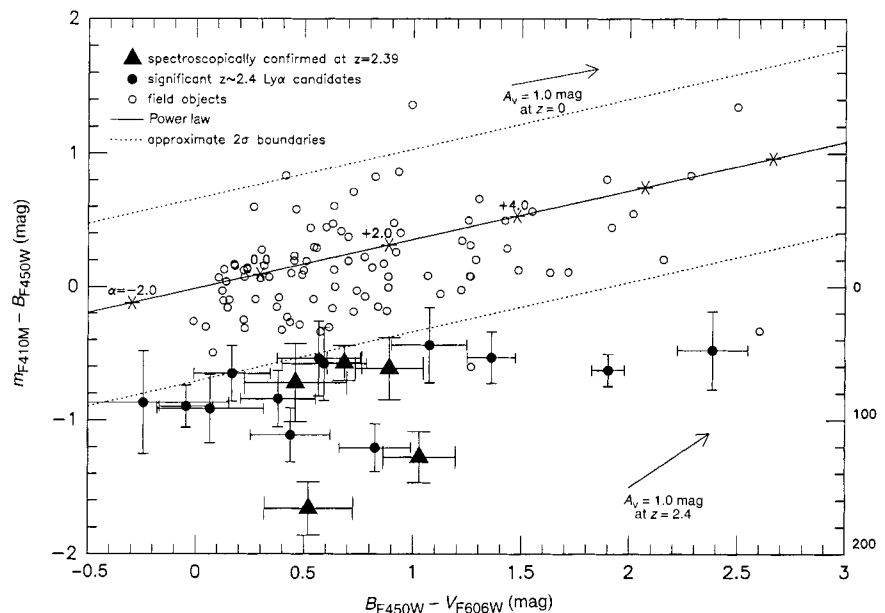
FIG. 2 Spectra of five of the $z \approx 2.39$ candidates, obtained using the Multiple Mirror Telescope (MMT). The spectra for 53W002 (object 6) and object 19 were taken with the ‘blue spectrograph’ and the 300 grooves per mm grating, and have been smoothed to ~ 20 Å resolution³¹. The spectra for objects 18, 12 and 8 were taken with the ‘red spectrograph’ and the 150 grooves per mm grating, and have been slightly smoothed to ~ 50 Å (ref. 7). The two candidates brightest in Lyman- α (objects 18 and 19) were discovered from ground-based narrow-band imaging⁷. All possible emission lines are labelled, as well as places where night-sky lines were poorly subtracted (indicated by ‘NS’).

enough that the original population of short-lived O and early B stars are gone, but shorter than the age of the dominant stellar population (late B and early A stars). Hence, there was indeed enough time for their mass distributions to settle into regular $r^{1/4}$ -like light profiles.

Assembling giant galaxies from fragments?

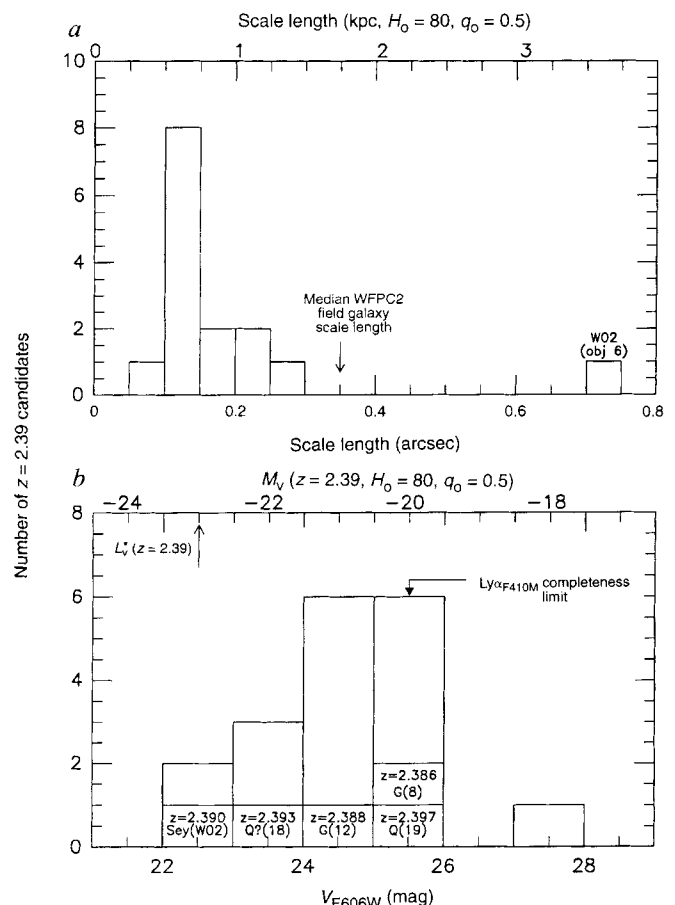
Most of the objects in Fig. 1 that were seen in V_{F606W} and I_{F814W} were also detected in B_{F450W} , limiting the maximum redshift sampled in this $\sim 2.5 \times 2.5$ arcmin field to typically $z \lesssim 3.5\text{--}4$, or else the expected Lyman limit would have significantly damped out the light in the B_{F450W} filter³⁶. We suggest that these sub-galactic-sized objects exist throughout the entire redshift range $z \approx 1\text{--}3.5$, and could have grown into the luminous giant galaxies (ellipticals and early-type spirals) that we see today through the process of repeated hierarchical merging⁶. An epoch-dependent merger rate proportional to $(1+z)^{2.5}$ would result in a time integral of $\sim 10\text{--}20$ mergers of compact objects from $z \approx 3.5$ to $z = 0$. This process of repeated merging would have to be largely complete by $z \approx 1$ if ellipticals and early-type spirals were in fact formed from such sub-galactic clumps, as their HST counts show little evolution since $z \lesssim 1$ (refs 9, 10, 20). Our 18 candidates could thus have merged to produce a few L^* galaxies today, given that objects separated by several hundred kiloparsecs will have many crossing times in the interval $z \approx 2.39$ to $z = 0$. The total

FIG. 3 The $m_{F410M} - B_{F450W}$ versus $B_{F450W} - V_{F606W}$ colour-colour diagram from 51 WFPC2 orbits on the 53W002 field, containing 115 objects which were detected in all three filters (limited by the shallower 11.25 hours taken with F410M). The solid line shows the expected relation for a featureless power-law spectrum ($F_{\nu} \propto \nu^{-\alpha}$), labelled with values of α , around which most of the general field objects (open circles) are distributed. Approximate 2σ error boundaries³¹ for the WFPC2 photometry are indicated by the dotted lines. Error bars are plotted only for objects $\geq 2\sigma$ below the power-law line (filled circles), which are significant candidate Lyman- α emitters at $z \approx 2.39$. Approximate implied rest-frame Lyman- α equivalent widths are indicated on the right-hand vertical axis. The five large filled triangles have a spectroscopic confirmation at $z \approx 2.39$ (see Fig. 2), and the two field objects (open circles) below the 2σ envelope in the region populated by the candidates have been spectroscopically confirmed not to be at $z \approx 2.39$. Both were found to be stars, the redder being an M star. Colours were determined from sufficiently large object apertures selected to be the same in all three filters. The sub-pixelated images were registered to well within 1 pixel. The sky was interpolated underneath the object aperture by fitting a sloped plane to the pixels unaffected by faint neighbours, hence correcting for any remaining small gradients in the flat-fielded images. Compared to automated object finding in the same field²⁰ the sky estimates are consistent to within 0.07% and the fluxes to within 0.05 mag. Therefore, including the WFPC2 zero-points⁵³ the photometry presented here is accurate to ~ 0.05 mag systematically, and to ~ 0.2 mag r.m.s. Both the observed and the rest frame ultraviolet reddening vectors⁵⁴ are indicated by the arrows, and suggest that the few reddest



candidates may have a visual absorption of $A_V \lesssim 2-3$ mag. These reddest candidates should be viewed with some caution, however, because of the slight dependence of the B_{F450W} magnitudes on $B_{F450W} - V_{F606W}$ colour, which increases towards redder colour⁵². (We corrected for this to first order, but higher-order terms may affect the reliability of the two $z \approx 2.39$ candidates with $B_{F450W} - V_{F606W} \gtrsim 1.5$ mag).

FIG. 4 a, Histogram of WFPC2 continuum scale lengths for the significant narrow-band redshifted Lyman- α emitting $z \approx 2.39$ candidates in the 53W002 field. Scale lengths were determined from the average of the half-light radii in B_{F450W} , V_{F606W} and I_{F814W} . Most $z \approx 2.39$ candidates have compact cores with $r_{HL} \lesssim 0.1-0.2$ arcsec or $\lesssim 0.5-1.0$ kpc, which is why they are relatively easy to detect with the HST; as the cores of these 'subgalactic clumps' are largely unresolved by the HST, they do not suffer much cosmological $(1+z)^4$ surface brightness dimming. (Their wings of course do, which is why any disks may disappear into the electronic background noise of the WFPC2 CCD.) b, Histogram of WFPC2 V_{F606W} luminosities for the significant $z \approx 2.39$ Lyman- α emitting candidates. The upper axis indicates their absolute magnitude distribution, assuming K -corrections for young stellar populations at $z \approx 2.39$ from Windhorst et al.³¹. (With BVI photometry, the K -corrections are straightforward, provided that their redshifts are known to be at $z \approx 2.39$ either from spectroscopy or their $m_{F410M} - B_{F450W}$ colours.) Weak AGN contributions were subtracted in a few cases as indicated, following ref. 32. The implied absolute magnitudes range from $M_V \approx -18$ to -23 mag, while the (evolving) value of L_V^* occurs at about $M_V = -23$ mag at $z \approx 2.39$, as indicated by the arrow (ref. 31). Some of the faintest candidates have $V_{F606W} \gtrsim 25-26$ mag (the figure becomes incomplete for $V_{F606W} \gtrsim 25.5$ mag, as shown by the second arrow, owing to the lower sensitivity in F410M compared to F450W; see Fig. 3 legend), so that it will require the concentrated efforts of the world's largest telescopes to confirm the redshifts of all $z \approx 2.39$ candidates spectroscopically. Given this completeness limit, the initial (luminous) mass spectrum of this group could thus be quite steep. The labelled boxes represent the five spectroscopically confirmed $z \approx 2.39$ candidates, with 'Sey' meaning Seyfert-like galaxy, 'Q' meaning quasar, and 'G' meaning galaxy (the numbers are the object identifications from Fig. 1).



V_{F606W} luminosity for the 18 objects is $M_V \approx -24.7$ mag at $z \approx 2.39$, which agrees rather well with the combined luminosities of a few typical L^* galaxies today of $M_V \approx -24.3$ mag (which includes the expected ~ -2 mag from their K -corrections plus evolution³¹).

The extremely narrow redshift distribution of the spectroscopically confirmed $z \approx 2.39$ objects ($\Delta z = 0.01$) as compared to the much broader width of the F410M filter ($\Delta z = 0.12$) leads one to question why objects at other redshifts in the range $2.30 < z < 2.42$ were not seen in our spectroscopic sample. The agreement of the space density ($\sim 0.027 \text{ Mpc}^{-3}$) and velocity dispersion ($\sim 290 \text{ km s}^{-1}$) of the objects in our sample with models based on the Press–Schechter theory (such as that shown in Fig. 1 of ref. 37), which predict the abundance of dark-matter haloes as a function of redshift, suggests that galaxies may have existed preferentially in groups or protoclusters at $z \approx 2.39$. In addition, ‘spikes’ that have been observed in the redshift distribution out to $z \approx 1$ may have also existed at some level out to $z \approx 1-3$, although the clustering amplitude at higher redshift would have to be lower according to recent deep galaxy surveys^{38,39} and most cold-dark-matter models. We can speculate that, in addition to groups or protoclusters, the existence of any such spikes could be due to the presence of some large-scale structure at high redshifts, as found to exist at somewhat lower redshifts⁴⁰⁻⁴². These structures may be in the form of ‘sheets’ thought to have been ‘frozen into’ the Universe shortly after the Big Bang, although this is very difficult to substantiate based on our images, which could only cover a small fraction ($\lesssim 1 \text{ Mpc}^2$) of such a structure. In redshift surveys out to $z \lesssim 1$ (refs 16, 40, 42–44), and most recently to $z \gtrsim 1$ (refs 18, 45, 46) with the Keck 10-metre telescope, often more than a few objects were found at very similar redshifts in these small fields, supporting the possible existence of clumpiness in the redshift distribution out to $z \gtrsim 1$. In addition, groups or other large structures have recently been found at redshifts comparable to our group²³.

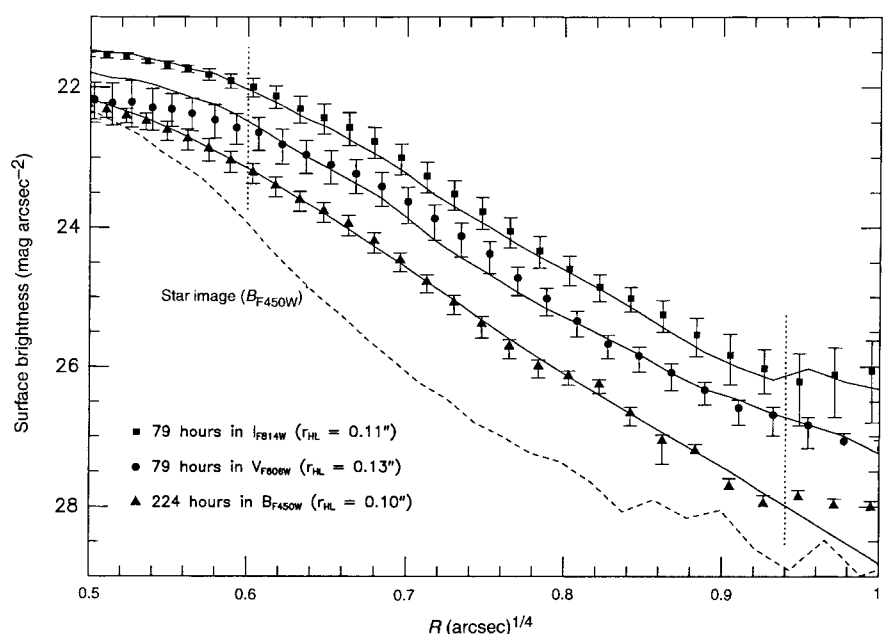
Some recent ground-based narrow-band imaging surveys²⁹ may

have missed any such groups or structures because the filters used were generally too narrow ($\sim 30 \text{ \AA}$) to detect one by pure chance unless some previous knowledge of the redshift already existed from, for example, one or more known quasars or radio galaxies. The WFPC2 medium-band filter F410M would sample typically one or two such redshift structures at $z \approx 2.39$ (and is not limited by sky-noise as most ground-based images are). Together with the extreme compactness of our objects, this explains our high detection rate with the HST.

Comparing our findings to those of Cowie, Hu and Songaila¹⁸, we find that about 5% of the faint blue objects in our HST field can be classified as ‘chain galaxies’, which is lower than the 20–50% estimated in their sample. We believe that such objects are probably the short-lived ($\lesssim 3 \times 10^8$ years out of a total of $\lesssim 5 \times 10^9$ years available for $z \gtrsim 1$) merger events among the many faint blue sub-galactic clumps, in which the gas is drawn out of the merging objects during the encounter^{6,47}.

We believe that a non-negligible fraction of the compact FBGs observed in modern deep galaxy surveys may be high-redshift sub-galactic-sized clumps such as those in our $z \approx 2.39$ group. The fraction of high-redshift objects has been found to increase significantly for $B \gtrsim 22$ mag (ref. 15). For $B \approx 26$ mag, the median redshift may be as high as ~ 1 with a substantial high-redshift ‘tail’^{18,19}. Hence the fraction of FBGs that are at high redshifts may increase as progressively fainter magnitudes are explored. Supporting this is recent work^{48,49} on the Hubble Deep Field, an area of the sky imaged by the HST and consisting of 342 exposures taken over 10 consecutive days, which has revealed large numbers of faint blue compact objects similar to our $z \approx 2.39$ candidates. It is thus possible that these sub-galactic clumps, and possibly a large fraction of the elusive primeval galaxies, may be hiding as many of these compact FBGs, and have escaped proper recognition from the ground until now because they are so small. This group of faint, compact star-forming subsystems at $z \approx 2.39$ could thus be the galactic building blocks long sought after by proponents of the ‘bottom-up’ galaxy formation models. Random

FIG. 5 Average light profiles of the intensity-weighted composite images of 14 of the $z \approx 2.39$ candidates in the B_{F450W} , V_{F606W} and I_{F814W} filters. To derive a mean intensity profile with the greatest dynamic range, intensity-weighted composite images of 14 compact and isolated candidates were produced in each filter, assuming that the objects are to first order all similar in shape and size at $z \approx 2.39$ (following M. Giallisco et al., manuscript in preparation). The total effective exposure times of the image stacks, therefore, are 14×5.7 hours in F606W and F814W, and 14×16 hours in F450W. The profiles are reliable for radii between the two vertical dotted lines, but are affected by the WFPC2 point-spread function and pixel-interpolation problems at smaller radii, and by errors in the sky-determination at larger radii. A stellar point-spread function profile is plotted (dashed curve) from the B_{F450W} data to illustrate that these compact clumps are indeed barely resolved by the HST. To measure the scale length of the mean observed profile, model profiles were convolved with an empirical point-spread function taken from a star in the images (for the under-sampled V_{F606W} and I_{F814W} images an additional ‘pyramid’ function was used to account for sub-pixel shifts among the objects, since shifting by interpolation introduces artefacts in undersampled images). The resulting best-fit profiles are indicated by the Solid lines. The B_{F450W} and F410M data were almost properly sampled, owing to 0.5 pixel substeps between individual exposure sequences, and the measurements were done on interleaved mosaics on 0.5-pixel centres. In each filter, the profiles are better fitted (in a χ^2 sense) by an $r^{1/4}$ -law than by a disk-like exponential over a range of almost five



magnitudes in surface brightness. The allowed range of half-light radii in WFC pixels ($\equiv 0.0996 \text{ arcsec}$) is: 0.8–1.1 for B_{F450W} (best-fit $r_{HL} = 1.00$); 1.1–1.4 for V_{F606W} ($r_{HL} = 1.30$); and 1.0–1.2 for I_{F814W} ($r_{HL} = 1.12$), corresponding to a mean of 0.11 arcsec or 0.54 kpc at $z \approx 2.39$. Despite differences in diffraction limits and sampling among the three broad-band filters, it is quite encouraging to find such comparable mean scale lengths.

WFPC2 observations through the F450W and F410M filters will tell whether or not our $z \approx 2.39$ group is unique in the early Universe. Finding additional similar objects would help to deter-

mine whether or not groups or protoclusters, or perhaps large-scale structures as seen at somewhat lower redshifts^{40,41}, are already common at $z \approx 2.39$. □

Received 9 February; accepted 26 July 1996.

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ACKNOWLEDGEMENTS. All image data were obtained with the NASA/ESA Hubble Space Telescope through the Space Telescope Science Institute (STScI), which is operated by the Association of Universities for Research in Astronomy, Inc. (AURA), under contract to NASA. The spectroscopic observations were obtained at the Multiple Mirror Telescope Observatory, a joint facility of the University of Arizona and the Smithsonian Institution. This research was supported by NASA/STScI.

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Role of *inscuteable* in orienting asymmetric cell divisions in *Drosophila*

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***Drosophila* neuroblasts and epithelial cells in the procephalic neurogenic region divide perpendicular to the surface, and segregate the proteins Numb and Prospero into the basal daughter cell. We demonstrate here that orientation of the mitotic spindle and correct localization of Numb and Prospero in these cells require the *inscuteable* gene. Moreover, ectopic expression of *inscuteable* in other epithelial cells leads to spindle reorientation. The Inscuteable protein localizes to the apical cell cortex before mitosis, suggesting that Inscuteable functions in establishing polarity for asymmetric cell division.**

A CELL can divide asymmetrically by segregating protein determinants into one of its two daughter cells¹. The membrane-associated protein Numb fulfils several criteria for such a determinant in the developing *Drosophila* nervous system^{2,3}. During the division of sensory organ precursor (SOP) cells in the developing peripheral nervous system (PNS), Numb localizes asymmetrically into a cortical crescent in prophase, and segregates into one of the two daughter cells in telophase^{3,4}. In the absence of Numb, this daughter cell is transformed into its sister. Overexpression of *numb* causes the opposite cell-fate transformation, suggesting that different concentrations of Numb can regulate the fates of the two

sibling cells. A similar function for Numb has been shown in the developing central nervous system (CNS)⁵.

Asymmetric segregation has also been described for the Prospero protein^{4,6,7}. Prospero is a nuclear transcription factor that is required for correct cell-fate specification and differentiation in the CNS, and for neuronal differentiation in the PNS^{8,9}. During prophase of mitosis, Prospero localizes into a cortical crescent. Throughout the rest of mitosis, Prospero colocalizes with Numb and segregates into the same daughter cell, where it enters the nucleus. Numb and Prospero localization are independent of each other^{4,7}. Localization of both proteins is tightly correlated with the

division axis, which is specified by the position of the two centrosomes and the orientation of the mitotic spindle (for a review of spindle orientation, see ref. 10). Both the Numb and Prospero crescents always overlie one of the two centrosomes^{4,7}. Positioning of the centrosome and localization of Numb and Prospero are independent events⁴, but the tight correlation suggests that both processes use the same positional information.

The *inscuteable* gene is expressed in cells that are known to localize Numb and Prospero. In neuroblasts the Inscuteable protein is asymmetrically localized to the apical side, opposite Numb and Prospero¹¹. Inscuteable contains a putative SH3 binding domain, a sequence motif commonly found in proteins that interact with the cytoskeleton. Shortly after gastrulation, *inscuteable* expression starts in the procephalic neurogenic region (PNR) of the ectoderm¹¹, when the ectoderm forms a

monolayered epithelium. Most cells in this epithelium divide parallel to the surface (that is, their mitotic spindle is oriented parallel to the surface), but the cells of the PNR, which will give rise to the larval brain, divide perpendicular to this surface¹². We show that both Numb and Prospero segregate into the basal daughter cell. Later, *inscuteable* is expressed in neuroblasts of the developing CNS¹¹. Neuroblasts also divide perpendicular to the epithelial plane but, in contrast to cells in the PNR, they delaminate from the ectoderm before mitosis¹³. During division, Numb and Prospero segregate into the basal daughter cell^{3,4,6,7}. The *inscuteable* gene is also expressed in SOP cells of the developing PNS¹¹ which asymmetrically localize Numb and Prospero^{3,4}.

We show here that *inscuteable* mutants are defective in both orientation of the mitotic spindle and localization of Numb and Prospero. We also demonstrate that ectopic expression of *inscuteable* in ectodermal cells outside the PNR leads to a reorientation of the mitotic spindle. Asymmetric localization of Inscuteable occurs before mitosis and precedes Numb and Prospero localization. Our results suggest that *inscuteable* functions to provide positional information for spindle orientation and asymmetric protein localization during mitosis.

Asymmetric localization of Inscuteable

In *Drosophila* neuroblasts, Inscuteable localizes to the apical cell

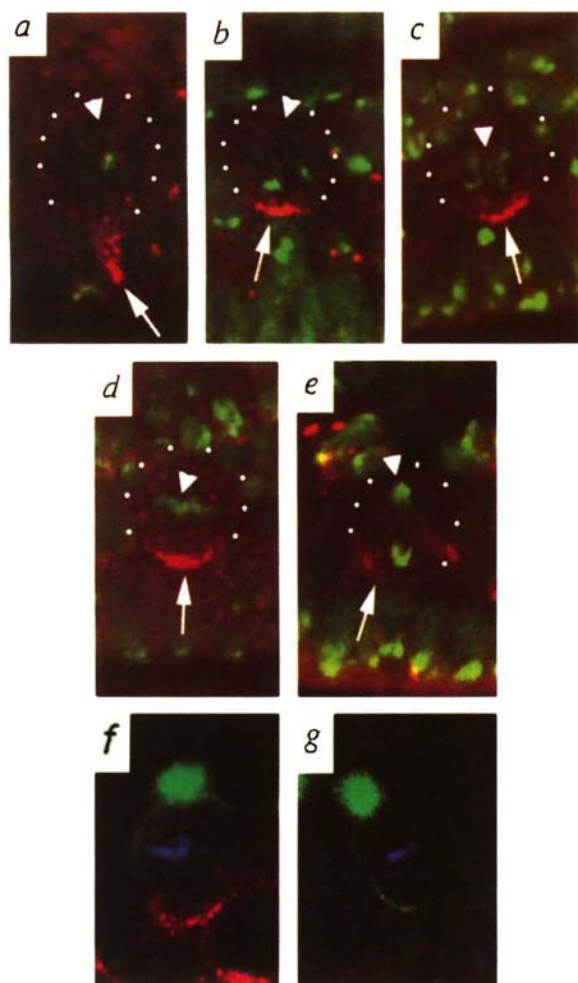


FIG. 1 Asymmetric localization of Inscuteable in neuroblasts precedes entry into mitosis and is dependent on the actin cytoskeleton. **a–e**, Paraxial optical sections through the neurogenic ectoderm of stage 9–10 embryos stained for Inscuteable protein (red, arrows) and DNA (green, arrowheads). Apical is down; neuroblasts are outlined by dots. **a**, Delaminating neuroblast. **b**, Delaminated late-interphase neuroblast. **c**, Prophase neuroblast. **d**, Metaphase neuroblast. **e**, Anaphase neuroblast. **f–g**, Cytochalasin D treatment disrupts Inscuteable localization. After treatment with $1 \mu\text{g ml}^{-1}$ cytochalasin D for 30 min, Inscuteable (red) becomes delocalized in metaphase cells (DNA in blue), whereas Prospero (green) is still localized into a crescent (**g**). Identical treatment without the drug does not disrupt Inscuteable localization (**f**). After cytochalasin treatment, Inscuteable was delocalized in 20 of 21 prophase or metaphase neuroblasts, but localized in 21 of 22 control neuroblasts. Prospero was asymmetrically localized in all of the drug-treated and control neuroblasts. However, the Prospero crescents were misoriented in 47% of the neuroblasts after cytochalasin treatment (14% in the controls).

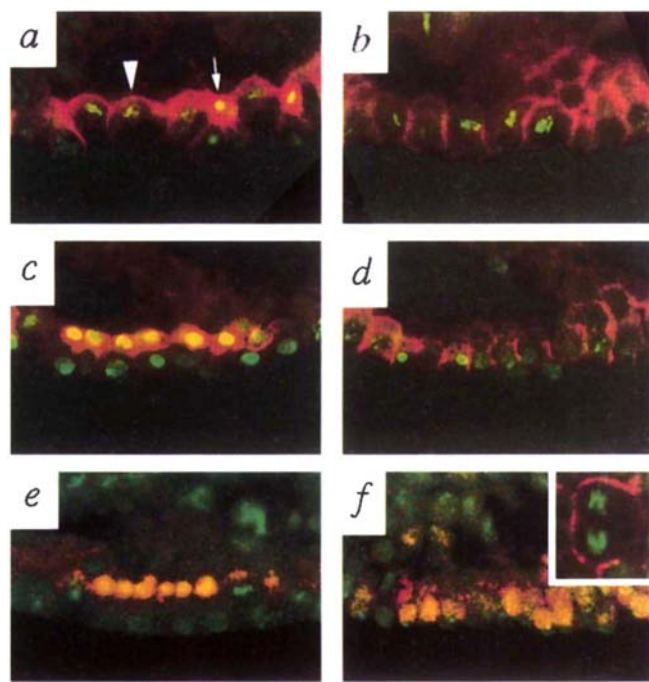


FIG. 2 Asymmetric localization of Numb and Prospero in the PNR requires *inscuteable*. **a–f**, Paraxial optical section through the procephalic neurogenic region (PNR) of wild-type (**a**, **c**, **e**) or *inscuteable*^{P72} mutant (**b**, **d**, **f**) embryos at late stage 9. Embryos were double labelled for Numb (red) and DNA (green) (**a–d**), or Prospero (red) and DNA (green) (**e**, **f**). **a–d**, Numb localization. **a**, **c**, In wild-type embryos, Numb was localized in a basal crescent during metaphase (arrowhead in **a**) and segregated into the basal daughter cell in telophase (**c**, and arrow in **a**). **b**, In mutant embryos no Numb crescents were detected in dividing cells of the PNR. **d**, After cell division, Numb could be detected in all cells of the PNR, indicating that the protein had not segregated preferentially into one of the two daughter cells. The position of the daughter cells was different as a result of the altered division plane (Fig. 6). **e**, **f**, Prospero localization. **e**, In wild-type embryos Prospero segregated into the basal daughter cell. **f**, In mutant embryos Prospero could be detected in all cells of the PNR after division, indicating that it did not segregate into one of the daughter cells. The inset shows a single dividing cell from the PNR during anaphase. Similar results were obtained for *inscuteable*^{P49} (not shown).

cortex¹¹, whereas Numb and Prospero localize to the basal cortex^{3,4,6,7}. Localization of Numb and Prospero is cell-cycle regulated. Their localization starts in late prophase and persists through metaphase and anaphase, and in telophase both proteins segregate into the basal daughter cell^{4,6,7}. We examined the cell-cycle dependence of Inscuteable localization in neuroblasts (Fig. 1a–e) by staining embryos at stage 9 and 10 with an anti-Inscuteable antibody¹¹ and a DNA stain¹⁴ to follow progression through mitosis. Inscuteable protein was first detected during delamination in a stalk that retains contact with the epithelial surface (Fig. 1a). In fully delaminated neuroblasts, Inscuteable formed a prominent crescent along the apical cell cortex (Fig. 1b). Shortly after delamination, neuroblasts enter mitosis and divide perpendicular to the epithelial plane^{7,15}. The apical Inscuteable crescent persisted through prophase and metaphase (Fig. 1c, d), but became delocalized in anaphase (Fig. 1e). In contrast to Numb and Prospero, Inscuteable did not preferentially segregate into one of the daughter cells. A similar time course of asymmetric localization of Inscuteable was also observed in the epithelial cells of the PNR (not shown).

We tested whether localization of Inscuteable was dependent on elements of the cytoskeleton. After disruption of actin filaments by treatment with cytochalasin D, Inscuteable crescents were no longer detectable in metaphase neuroblasts (Fig. 1f, g). In the same cells, Prospero was still asymmetrically localized (Fig. 1g), suggesting that the effect was specific and not due to cell death or damage. However, the position of the Prospero crescent was incorrect in 47% of the scored neuroblasts. No defects in Inscuteable localization could be observed after destruction of microtubules with colcemid (data not shown). Thus Inscuteable

localization is actin dependent, and precedes both mitosis and the asymmetric localization of Numb and Prospero.

Localization of Numb and Prospero

Inscuteable expression occurs in cells that are known to localize Numb and Prospero¹¹, and starts in ectodermal cells of the PNR¹¹. Before division, these cells are morphologically indistinguishable from other ectodermal cells. In contrast to most other ectodermal cells, however, they divide perpendicular to the epithelial plane¹². During this division, both Numb and Prospero localized asymmetrically and segregated into the basal daughter cell (Fig. 2a, c, e). Prospero expression at this stage was specific to the PNR, whereas Numb was present, but not asymmetrically localized, in ectodermal cells outside the PNR (data not shown). In embryos carrying *inscuteable* null alleles, Numb and Prospero were not asymmetrically localized during mitosis in the PNR (Fig. 2b, d, f). Both proteins were localized to the cell membrane, but no Numb or Prospero crescents could be detected ($n = 50$), and the proteins segregated into both daughter cells. Prospero expression remained specific to the PNR (Fig. 2f and data not shown), suggesting that these cells were not simply transformed into cells of other ectodermal regions.

Defects in Numb and Prospero localization were also found in dividing neuroblasts of *inscuteable* mutant embryos (Figs 3 and 4). Normally, Numb and Prospero colocalize into basal crescents during mitosis and segregate into the basal daughter cell (Fig. 3a–c, g–i). In *inscuteable* null mutants, both Numb and Prospero were mislocalized. Prospero was found around the circumference of the cell in 24% of dividing neuroblasts. In those neuroblasts where the Prospero crescents did form (76%), their orientation was ran-

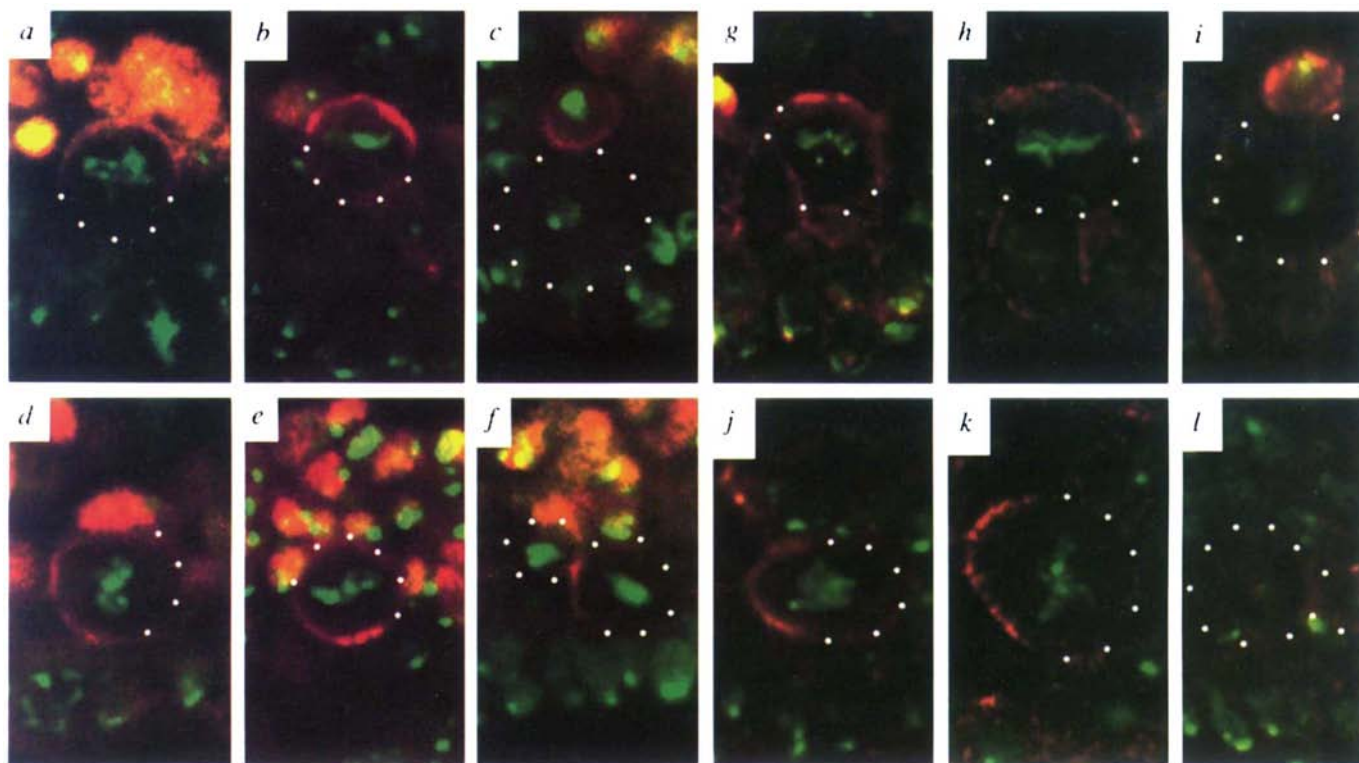


FIG. 3 Basal localization of Prospero and Numb in neuroblasts requires *inscuteable*. Parasagittal optical sections through the ventral ectoderm of stage 10–11 embryos showing different neuroblasts (delineated by dotted line) at various stages of division. Apical is at the bottom. a–c, Prospero (red) and DNA (green) in wild-type neuroblasts. During mitosis (a–c), Prospero was localized basally (up) at prophase (a) and metaphase (b), and segregated into the basally located ganglion mother cell at telophase (c). Note that divisions were oriented apico-basally. d–f, Prospero (red) and DNA (green) in *inscuteable*^{P49} neuroblasts. A Prospero crescent was found

at random, non-basal locations (d, e, f), or Prospero was delocalized and found throughout the cortex (not shown). Note that division was misoriented and Prospero sometimes fails to become localized to the ganglion mother cell as in f. g–i, Numb (red) and DNA (green) in wild-type neuroblasts. Like Prospero, Numb was localized basally during mitosis, and segregated into the ganglion mother cell (i). j–l, Numb (red) and DNA (green) in *inscuteable*^{P49} neuroblasts. Numb, like Prospero, was not always basally localized (j–l), and division was similarly misoriented (l). Similar results were obtained for *inscuteable*^{P72}.

domized (Figs 3d–f and 4b); similar results were obtained for localization of Numb in *inscuteable* mutant embryos (Fig. 3j–l). However, Numb and Prospero were still colocalized in 90% ($n = 20$) of the mutant neuroblasts. In contrast, no defects were seen for Inscuteable localization in *numb* and *prospero* mutants (data not shown). Our results demonstrate that correct localization of Numb and Prospero in neuroblasts and cells of the PNR requires *inscuteable*.

Spindle orientation

Localization of Numb and Prospero is tightly correlated with the orientation of the mitotic spindle⁴. To determine whether *inscuteable* is involved in (and coordinates) both processes, we analysed the orientation of the mitotic spindle in dividing neuroblasts of *inscuteable* mutants. In wild-type neuroblasts, one of the two centrosomes migrates to the basal side after delamination⁷ and the mitotic spindle is set up perpendicular to the epithelium. The orientation of the mitotic spindle in dividing neuroblasts of *inscuteable* mutant embryos was analysed by double staining for

centrosomes and DNA (Fig. 5). Although the centrosomes still migrated to opposite poles of the cells, the mitotic spindle was not oriented along the apical basal axis in most *inscuteable* mutant neuroblasts (Fig. 5c, d). Statistical analysis showed that spindle orientation was less ordered in mutant embryos (Fig. 4a). Moreover, the tight correlation found in wild-type cells between the position of the Numb and Prospero crescents and the position of the spindle pole was disrupted in mutant neuroblasts (Fig. 4c, d).

Defects in spindle orientation were also detected in the PNR of the ectoderm (Fig. 6a–d). In contrast to neuroblasts, the mitotic spindle is established parallel to the epithelial surface in cells of the PNR. Early in mitosis, however, the spindle rotates by 90° leading to a division along an apical–basal axis¹² (Fig. 6a, b). Double staining of cells in the PNR for β -tubulin and DNA revealed that this reorientation of the mitotic spindle did not occur in *inscuteable* mutants (Fig. 6c, d). Spindles were oriented parallel to the surface, even in cells late in mitosis (Fig. 6d), and cells divided parallel to the epithelial plane. Our results demonstrate that *inscuteable* is required for spindle orientation perpendicular to the surface in both neuroblasts and cells of the PNR.

Spindle reorientation

To test whether *inscuteable* is also sufficient for reorientation of the mitotic spindle, *inscuteable* was misexpressed in ectodermal cells outside the PNR (Fig. 6e–j). The GAL4 system¹⁶ was used to express *inscuteable* ectopically in the expression pattern of the pair-rule gene *hairy* (see Fig. 6 legend and methods). In control stage-11 embryos, ectodermal cells in abdominal segments did not express *inscuteable* and divided parallel to the epithelial surface (Fig. 6e, f, i). In embryos where *inscuteable* was ectopically expressed in these abdominal ectodermal cells, the ectopically produced Inscuteable protein was found on the apical cell cortex (Fig. 6j); after ectopic expression of *inscuteable*, these cells reoriented their mitotic spindle and divided perpendicular to the epithelial surface (Fig. 6g, h, j). Thus *inscuteable* expression in ectodermal cells is sufficient to reorient the mitotic spindle and the division plane. Even though the division plane was changed, cell divisions did not become asymmetric after ectopic *inscuteable* expression (data not shown). Numb, which is expressed in all cells of the ectodermal epithelium in both control and transgenic embryos, was not asymmetrically localized, and the two daughter cells were similar in size and shape. Ectopic expression of *inscuteable* was not sufficient to turn on Prospero expression, indicating that ectodermal cells were not transformed into cells

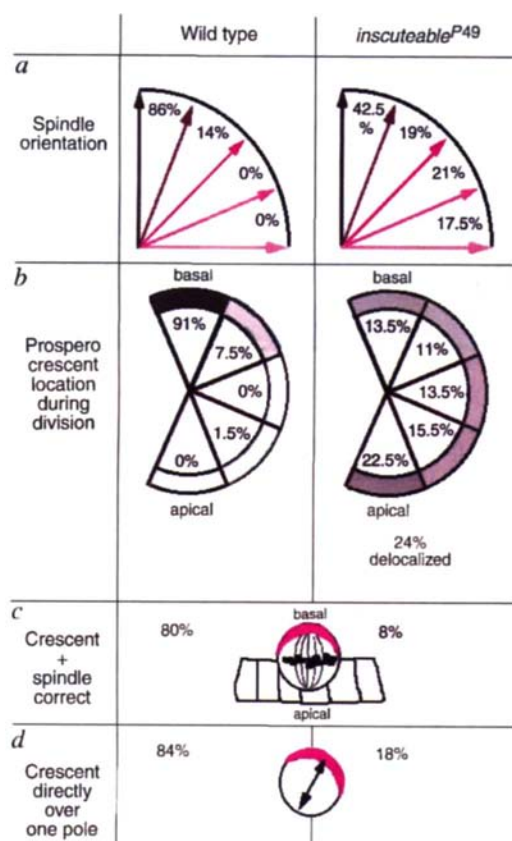


FIG. 4 Prospero localization, spindle orientation and their correlation are perturbed in dividing mutant neuroblasts. **a**, In wild-type neuroblasts most mitotic spindles were oriented perpendicular to the plane of the overlying ectoderm ($n = 50$); in *inscuteable*^{P49} embryos, spindle orientation was less ordered ($n = 52$). **b**, Prospero crescents in wild-type neuroblasts were primarily basally oriented ($n = 68$); in mutant neuroblasts, Prospero often failed to form a crescent (24%), and crescents that did form were oriented randomly ($n = 111$). **c**, In 80% of wild-type neuroblasts ($n = 50$) the division axis was apical–basal and the Prospero crescent was localized directly over the basal pole; in *inscuteable*^{P49} only 8% of the neuroblasts showed this configuration ($n = 52$). **d**, In mutant neuroblasts, the orientation of the division axis and the localization of the Prospero crescent were no longer tightly coupled. In the wild type, the Prospero crescent was localized directly over one spindle pole (regardless of the division axis) in 84% of dividing neuroblasts ($n = 50$), as against only 18% ($n = 52$) in mutants. We expect a similar decoupling of the position of the Numb crescents to the orientation of the division axis because Prospero and Numb crescents remain colocalized in 90% of the mutant neuroblasts (see text).

FIG. 5 Correct orientation of the mitotic spindle in neuroblasts requires *inscuteable*. **a–d**, Parasagittal optical sections through the ventral ectoderm of stage 10–11 wild-type (**a**, **b**) and *inscuteable*^{P49} (**c**, **d**) embryos, double stained for centrosomes³¹ (red, arrows) and DNA (green), showing the orientation of mitosis in dividing neuroblasts. Apical is down. **a**, **b**, In wild-type embryos spindles were oriented apical–basal or nearly apical–basal in all neuroblasts. **c**, **d**, In *inscuteable* mutants, spindles were oriented more randomly (see Fig. 4a). Note that in *inscuteable* mutants, as in wild type, the centrosomes migrated to opposite poles of the cell.

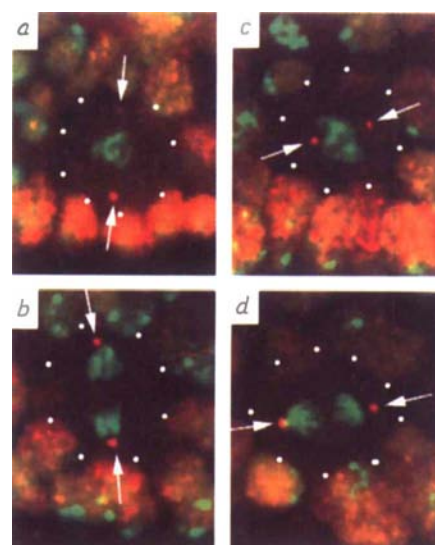
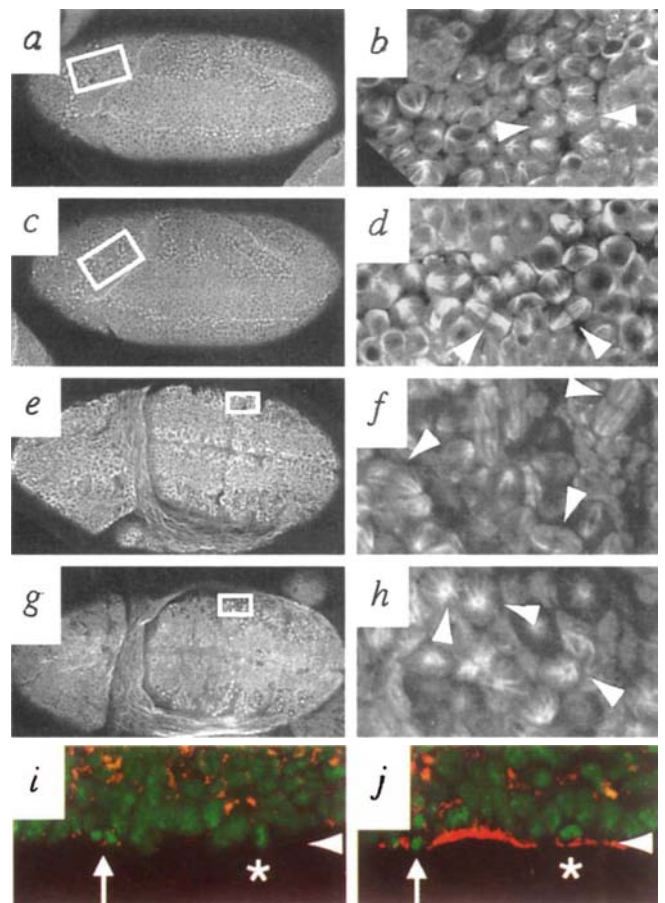


FIG. 6 Spindle orientation in ectodermal cells of the PNR requires *inscuteable*, and ectopic expression of *inscuteable* leads to reorientation of the spindle. **a–d**, Surface views of stage-9 wild-type (**a**, **b**) and *inscuteable*^{P72} (**c**, **d**) embryos stained for β -tubulin. **a**, **b**, Wild-type cells of the PNR (boxed in **a** and enlarged in **b**) divided perpendicular to the epithelial surface¹² (arrowheads); **c**, **d**, cells in the PNR of mutant embryos of the same stage (boxed in **c** and enlarged in **d**) did not rotate their spindle and divided parallel to the surface (arrowheads). **e–h**, Surface views of stage-11 control embryos (**e**, **f**) and embryos ectopically expressing *inscuteable* (**g**, **h**) stained for β -tubulin. The boxed areas in **e** and **g** are enlarged in **f** and **h**, respectively. **e**, **f**, In control (and in wild-type) embryos, cells of the lateral ectoderm do not express *inscuteable* and divided parallel to the surface (arrowheads in **f**). **g**, **h**, Embryos carrying both the *hairy*-GAL4¹⁶ and the UAS-*inscuteable* transgenes expressed *inscuteable* in a pair-rule expression pattern. Cells ectopically expressing *inscuteable* rotated their mitotic spindle and divided perpendicular to the surface (arrowheads in **h**). **i**, **j**, Parasagittal optical sections through adjacent ectodermal regions of an embryo carrying the *hairy*-GAL4 (ref. 16) and the UAS-*inscuteable* transgene. Apical is down. Inscuteable (red) and DNA (green) are shown, metaphase (asterisks) and anaphase (arrows) cells are marked. Settings on the confocal and image processing were identical for **i** and **j**. **i**, In ectodermal regions not expressing Inscuteable (arrowhead), cells divided parallel to the surface (arrow). **j**, Ectopically expressed Inscuteable localized to the apical surface (arrowhead) in interphase and metaphase cells, but became delocalized in anaphase (arrow). In regions ectopically expressing *inscuteable*, cells divided perpendicularly to the epithelial surface (arrow).



of the PNR. Older embryos showed no obvious epithelial defects after *inscuteable* misexpression. No changes in cell number and cell density were detected in their epidermis, suggesting that both daughter cells were later reincorporated into the epithelium.

Our results demonstrate that, in ectodermal cells, *inscuteable* expression is necessary and sufficient for reorientation of the division plane, and is necessary but not sufficient for basal localization of Numb and Prospero.

Discussion

Asymmetric localization of Numb and Prospero during mitosis is tightly correlated with, but independent of, the orientation of the mitotic spindle. Here we show that *inscuteable* is required for both Numb and Prospero localization and spindle orientation, as well as their normally tight correlation. The apical cortical localization of Inscuteable protein occurs before mitosis and precedes the basal localization of Numb and Prospero. In *inscuteable* null mutants, neuroblasts divide in more random orientations and fail to correctly localize Numb and Prospero. Epithelial cells of the PNR, which normally divide perpendicular to the epithelial surface, fail to rotate their mitotic spindle in mutants; they divide parallel to the surface and fail to localize Numb and Prospero asymmetrically. Ectopic *inscuteable* expression in ectodermal cells outside the PNR leads to reorientation of the mitotic spindle. Our results suggest that asymmetric localization of Inscuteable during interphase functions to establish positional information for both spindle orientation and asymmetric localization of Numb and Prospero during mitosis.

In *Caenorhabditis elegans*, spindle orientation in the AB and P1 cells, the two daughter cells of the first embryonic cell division, is thought to depend on the absence or presence of a cortical microtubule attachment site^{17–19} (reviewed in ref. 20). In the P1 cell, microtubules originating from one of the two centrosomes contact this cortical site, shorten and thereby pull the attached centrosome and the mitotic spindle towards the cortical site¹⁸. This leads to division of the P1 cell along the anterior–posterior axis of the embryo, while the AB cell, which does not have a cortical site, divides perpendicular to this axis. Similarly, experiments in yeast have demonstrated that spindle orientation seems to be mediated by interactions between astral microtubules and the cortical actin cytoskeleton²¹ (reviewed in ref. 22). Assuming that a similar mechanism reorients mitotic spindles in the *Drosophila* ectoderm, Inscuteable could be involved directly or

indirectly in providing an attachment site for microtubules. Mutations that affect the orientation of the mitotic spindle have been described in other organisms, for example mutations in the *C. elegans* maternal genes *par1*–*par6* (refs 23–25), and the *Arabidopsis* genes *FASS* (ref. 26), *ton1* or *ton2* (ref. 27; see ref. 28 for review). However, the genetic evidence suggests that a functional parallel between these genes and *inscuteable* seems unlikely.

The *inscuteable* gene is required for correct spindle orientation in both neuroblasts and the PNR. In contrast to ectodermal cells of the PNR, which divide parallel to the surface in the absence of *inscuteable*, mitotic spindles in mutant neuroblasts can assume intermediate orientations that are neither perpendicular nor parallel to the surface. This might be explained by the different ways in which the mitotic spindle is set up in these two cell types. Ectodermal cells in the PNR set up their spindle parallel to the epithelial surface and then rotate it by 90°; in contrast, neuroblasts set up their spindle perpendicular to the epithelial plane after one of the two centrosomes migrates to the basal side of the cell⁷, suggesting that cortical contact is made before centrosome separation and is required to keep one centrosome in place. In the absence of this anchoring mechanism, the resulting spindle would be misoriented. Thus the different *inscuteable* phenotypes in neuroblasts and epidermal cells can be explained if Inscuteable were to have the same underlying cellular function in either positioning or establishing a cortical site for movement or anchoring of one of the two centrosomes.

As well as having defects in spindle orientation, *inscuteable* mutant embryos are also defective in the asymmetric localization of Numb and Prospero. These defects are not secondary effects of incorrect spindle orientation, because Numb and Prospero localization are independent of the mitotic spindle both in neuroblasts⁸ and the PNR (data not shown; see Fig. 4c, d). Both proteins

become localized to the basal cell cortex in late prophase, when Inscuteable is localized to the apical cortex. The different sub-cellular localization of the proteins makes a direct interaction between Inscuteable and Numb or Prospero unlikely. Inscuteable could function by excluding Numb and Prospero from one half of the cell cortex, but we favour the possibility that other proteins could redistribute to the basal cell cortex in response to asymmetric localization of Inscuteable to the apical side. During mitosis, these proteins might then acquire affinity for Numb and Prospero. Our observation that the ectopic expression of *inscuteable* is insufficient to induce Numb localization suggests that other proteins which are not present in the ectoderm outside the PNR, might be involved in the asymmetric localization.

The absence of *inscuteable* leads to defects in Numb and Prospero localization in both neuroblasts and cells of the PNR, but no defects were readily detectable in dividing SOP cells. Both proteins were still localized into a crescent over one spindle pole (data not shown). Unlike neuroblasts and cells of the PNR, SOP cells generally divide parallel, not perpendicular, to the epithelial surface²⁹ (data not shown). However, different SOP cells have different spindle orientations relative to the anterior–posterior and dorsal–ventral axes of the embryo, making it difficult to determine whether in *inscuteable* mutants these spindles and their associated crescents are correctly oriented. It thus remains possible that SOP cell divisions are misoriented, leading to PNS defects in *inscuteable* mutants¹¹. Alternatively, it is possible that Inscuteable functions only in orienting divisions along the apical–basal axis, and a second cellular mechanism is involved in directing cell divisions parallel to the epithelial plane, such as the division of SOP cells. Several genes that are required for correct tissue polarity within the epithelial plane³⁰ are candidate components of this second mechanism. The observed PNS defects in *inscuteable* mutants¹¹ could then be explained by a second function of the gene which is suggested by the non-cell division-related phenotypes reported in ref. 11 and is not related to orientation of cell division.

Cytochalasin D treatment disrupts the localization of Inscuteable, but not of Numb and Prospero, in mitotic neuroblasts. Numb and Prospero are still localized after treatment with cytochalasin D, but their crescents are frequently misoriented⁴ (Fig. 1). Absence of Inscuteable can lead to a similar misorientation of Numb and Prospero crescents in neuroblasts. Thus the effect of cytochalasin treatment on Numb and Prospero localization might be an indirect consequence of disrupted Inscuteable localization in these experiments. Both the actin dependence of Inscuteable localization and the presence of a putative SH3 binding domain suggest a tight connection between Inscuteable localization and cytoskeletal asymmetry. We wondered where the information for asymmetric localization of Inscuteable comes from. Experiments in yeast have shown that orientation of asymmetric cell divisions can rely on determinants that are left from the previous cell

division (for a review see ref. 22). In contrast, Inscuteable localizes to the apical cortex of ectodermal epithelial cells and neuroblasts, irrespective of the orientation of their previous division, suggesting that the localization responds to the apical–basal polarity of the epithelium. Future experiments will determine whether Inscuteable acts by translating epithelial polarity into positional information that can be used for orientation of cell divisions and asymmetric protein localization during mitosis. □

Methods

Immunocytochemistry and confocal microscopy. Wild-type (Canton-S), *inscuteable*^{P72} and *inscuteable*^{P49} (two null alleles of *inscuteable*¹¹) embryos were fixed and stained with Inscuteable¹¹, Numb³, Prospero^{6,7} and Rb188 (a centrosomal marker)³¹ antibodies for immunofluorescence, essentially as described^{4,11}. Staining with β -tubulin antibody (Amersham) was performed essentially as described³². DNA was visualized either with propidium iodide³³ (Figs 2 and 6) or by mounting embryos in a 1:1 mixture of phenylene diamine DNA stain¹⁴ (Figs 1, 3 and 5) and Vectashield (Vector). Secondary antibodies were obtained from Molecular Probes. Mutant chromosomes were balanced with CyO, *P[w⁺ftz-lacZ]*, and homozygous mutant embryos were identified by double staining with β -gal antibodies (Promega and Cappel) based on the absence of *lacZ* expression. Confocal microscopy was done on a Biorad MRC 600, using Adobe Photoshop 3.0 for image processing.

Cytochalasin treatment. Cytochalasin D (Sigma) treatments were performed by incubating dechorionated Canton-S embryos in 1:1 heptane: Schneider's insect medium (SIGMA), without (addition of 0.001 volume DMF) or with 1 μ g ml⁻¹ cytochalasin D (addition of 0.001 volume 1 mg ml⁻¹ cytochalasin D in DMF) for 30 min. The Schneider's medium was then replaced by fixation buffer, and the embryos were fixed as described¹¹ and stained for Prospero⁷, DNA¹⁴ and Inscuteable (using a rabbit anti-Inscuteable antibody raised with a previously described Inscuteable fusion protein¹¹). Embryos were mounted in Vectashield. Phalloidin staining reveals an intact actin cytoskeleton in control embryos, but disruption of the actin cytoskeleton after inclusion of cytochalasin D in the treatment (not shown).

Assessment of spindle orientations and protein localization. Wild-type and *inscuteable*^{P49} embryos were stained with a centrosome-specific antiserum³¹ and a DNA stain¹⁴, and analysed by confocal microscopy. Neuroblasts were photographed and the angle of the division, according to the position of the centrosomes and DNA, was assessed with respect to the plane of the epithelium. Prospero localization was estimated in *inscuteable*^{P49} and wild-type (Canton-S) embryos by staining dividing neuroblasts for Prospero and DNA simultaneously. For Fig. 4c, d, neuroblasts in metaphase or in later stages of division were tabulated. Similar results were obtained for *inscuteable*^{P72} (data not shown).

GAL4-mediated misexpression of *inscuteable*. The *UAS-inscuteable* transgene was constructed by inserting a *HindIII*–*NotI* fragment of the *inscuteable* cDNA¹¹ into the vector pUAST¹⁶. Transgenic flies were generated as described³⁴. A heterozygous insertion on the third chromosome was used. Virgin female flies carrying the heterozygous P-element insertion IJ3, which expresses GAL4 in the *hair* expression pattern¹⁶, were crossed to wild-type males (control) or males carrying the *UAS-inscuteable* transgene. Embryos from both crosses were aged to stage 11, fixed and stained for β -tubulin and DNA or for Inscuteable¹¹ and DNA³³ as described above. No changes in spindle orientation, compared with wild type, were observed in the control. When *UAS-inscuteable* males were used, however, one-quarter of the embryos displayed the spindle reorientation phenotype shown in Fig. 6.

Received 1 July; accepted 31 July 1996.

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ACKNOWLEDGEMENTS. We thank P. O'Farrell and members of the Jan and Chia laboratories for discussions; D. Schneider, C. P. Shen, A. Sil, F. Sprenger, M. Vetter, X. Yang, P. Li and B. Murugasu-Oei for comments on the manuscript; X. Yang and M. Zavortink for help with the cytochalasin experiment; and E. Spana, C. Q. Doe, D. Glover and J. A. Campos-Ortega for reagents. The Chia laboratory is supported by the National Science and Technology Board (Singapore) and the European Community; J.A.K. is supported by an EMBO postdoctoral fellowship; L.Y.J. and Y.N.J. are HHMI investigators.

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Self-gravity as an explanation of the fractal structure of the interstellar medium

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THE gas clouds of the interstellar medium have a fractal structure, the origin of which has generally been thought to lie in turbulence^{1,2}. The energy of turbulence could come from galactic rotation at large scale, then cascade down to be dissipated on small scales by viscosity^{5,14}; it has been suggested that such turbulence helps to prevent massive molecular clouds from collapsing in response to their own gravity^{15,16}. Here we show that, on the contrary, self-gravity itself may be the dominant factor in making clouds fractal. We develop a field-theory approach to the structure of clouds, assuming them to be isothermal, and with only gravitational interactions; we find that the observed fractal dimension of the clouds arises naturally from this approach. Although this result does not imply that turbulence is not important, it does demonstrate that the fractal structure can be understood without it.

The interstellar medium (ISM) is an ensemble of gas clouds and dust, composed mainly of hydrogen (either atomic H I or molecular H₂) and helium (~25% by mass), with other elements present in trace amounts (dust is only ~1% in mass). The bulk of the ISM is distributed in cold clouds (temperature, $T \approx 5$ –50 K), forming a very fragmented and clumpy structure, which is confined to the galactic plane of spiral galaxies.

For at least two decades, radioastronomy line observations (H I at a wavelength of 21 cm and CO at a wavelength of 2.6 mm for the major lines) have told us that the ISM is composed of a hierarchy of structures, with masses from about $1 M_{\odot}$ to $10^6 M_{\odot}$. Structures have been observed in the ISM, with sizes from 10^{-4} pc (20 AU or 3×10^{14} cm) to 100 pc. The largest of these structures are giant molecular clouds or complexes, thought to be the largest self-gravitating structures in the Galaxy. Above 100 pc, larger structures would be destroyed by galactic shear. The accumulation of observations at many scales, and with many tracers of the ISM (CO and its isotopes, HCN, CS, NH₃, H I and dust, as in Fig. 1) revealed that the interstellar medium obeys power-law relationships between size (R), mass (M) and internal velocity dispersion (Δv) (see for example refs 1, 3):

$$M(R) \propto R^{d_H} \text{ and } \Delta v \propto R^q \quad (1)$$

These apply across the entire observed range of structure sizes and masses, with Hausdorff dimension (d_H) and the power q in the ranges

$$1.5 \leq d_H \leq 2 \text{ and } 0.3 \leq q \leq 0.5 \quad (2)$$

Structures appear virialized at any scale: the scaling laws obey the relationships $\Delta v^2 \propto GM/R$, or equivalently $q = (d_H - 1)/2$.

Here we apply, for the first time, field theory and Wilson's approach to critical phenomena⁴, to the problem of a gravitational gas in statistical equilibrium. We consider a gas of non-relativistic particles in thermal equilibrium at temperature T interacting with each other through newtonian gravity. We work in the grand canonical ensemble, allowing for a variable number of particles N . The grand partition function of this system of particles with masses m and phase-space coordinates $\mathbf{p}$ and $\mathbf{q}$, can be written as

$$\mathcal{Z} = \sum_{N=0}^{\infty} \frac{z^N}{N!} \int \dots \int \prod_{i=1}^N \frac{d^3 p_i d^3 q_i}{h^3} e^{-\frac{H_N}{kT}}$$

where

$$H_N = \sum_{i=1}^N \frac{p_i^2}{2m} - Gm^2 \sum_{1 \leq i < j \leq N} \frac{1}{|\mathbf{q}_i - \mathbf{q}_j|} \quad (3)$$

where G , h and k are Newton's gravitational constant, Planck's constant and the Boltzmann constant respectively, and z is the fugacity $z = e^{\frac{\mu}{kT}}$; μ is the chemical potential. By transforming this expression with a functional integral^{5,6}, it can be shown that this system is exactly equivalent to the theory of a scalar field $\phi(\mathbf{x})$ (the detailed derivation will be found in d.V., N.S. and F.C., manuscript in preparation); the grand canonical partition function $\mathcal{Z}$ can be expressed as a functional integral

$$\mathcal{Z} = \iint \mathcal{D}\phi e^{-S[\phi]}$$

with

$$S[\phi] \equiv \frac{1}{T_{\text{eff}}} \int d^3 x \left[\frac{1}{2} (\nabla \phi)^2 - \mu e^{\phi(\mathbf{x})} \right] \\ T_{\text{eff}} = 4\pi \frac{Gm^2}{kT} \quad \mu^2 = \frac{\pi^{5/2}}{h^3} z G (2m)^{7/2} \sqrt{kT} \quad (4)$$

The parameter μ corresponds to the inverse of the Jeans length $\mu = \sqrt{\frac{12}{\pi} \frac{1}{d_j}}$. The stationary points of the action S are given by the equation for the dimensionless field ϕ :

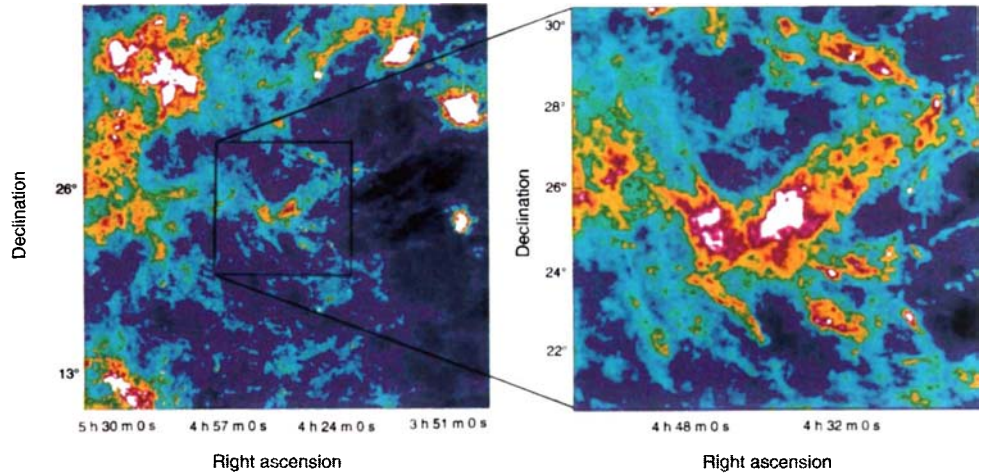
$$\Delta \phi = -\mu^2 e^{\phi} \quad (5)$$

This is exactly the equation satisfied by the gravitational field $U = -\frac{kT}{m} \phi$ of a perfect isothermal gas. Indeed, the equation of state combined with the equation for hydrostatic equilibrium, yields $\rho = \rho_0 e^{-\frac{kT}{m} U}$. The application of the Poisson equation leads to (5), provided that $\rho_0 = z(2\pi m k T)^{3/2}$. This leads to the well-known solution of the isothermal sphere, and small fluctuations around the stationary point have been studied⁷. In terms of the scalar field ϕ , the particle density can be expressed as $\langle \rho(\mathbf{r}) \rangle = -\frac{1}{T_{\text{eff}}} \langle \Delta \phi(\mathbf{r}) \rangle = \frac{\mu^2}{T_{\text{eff}}} \langle e^{\phi(\mathbf{r})} \rangle$ where $\langle \dots \rangle$ indicates the functional average over $\phi(\cdot)$ with statistical weight $e^{-S[\phi]}$.

The term $-\mu^2 e^{\phi}$ which makes S unbounded from below reflects the short-distance gravitational attraction. We limit the newtonian forces at short distances as the interparticle interaction is not purely gravitational at short distances. The ISM in isothermal conditions (that is, the ISM in contact with the cosmic background radiation which acts as a heat bath), is unstable owing to Jeans instability at any scale⁸; fragmentation occurs down to the scale where the coupling with the thermal bath breaks down; at this point the system becomes adiabatic instead of isothermal. The scale at which this transition occurs is that of the smallest possible fragments. Our isothermal gravitational gas model therefore has a natural limit at this scale.

Having derived the N -body, gravitational, scalar field, first we look at a perturbative method. We note that $S[\phi]$ has no constant stationary points, except $\phi_0 = -\infty$. In order to compute perturbations around ϕ_0 , we introduce a small constant term into the density, so that $\phi_0 = \log \delta$ is finite for non-zero values of the constant δ (d.V., N.S. and F.C., manuscript in preparation). The perturbative development in terms of the dimensionless coupling constant $g = \sqrt{\mu T_{\text{eff}}}$ reveals that the field ϕ is massless; the two-point density correlation function decreases like r^{-2} at large distances. Therefore, the theory remains critical, for large ranges of values of the physical parameters. As we consider the gas inside a large sphere of radius R ($R \leq 100$ pc, since other forces are involved at greater scales), no divergences appear at large

FIG. 1 Image of the interstellar medium near the Taurus region at 100 μm wavelength, obtained with the IRAS satellite (the continuum emission comes from dust heated by the interstellar radiation field, in this low star-forming region). The linear size of the box is 30° , and the pixel is 6 arcmin. An enlargement by a factor of three in scale of the central region is shown: the overall fragmented structure of the medium remains unchanged. This self-similar structure has been confirmed by the compilation of many other observations at widely different scales, revealing the fractal structure of the ISM.



radii. It is more informative to study the perturbative development around the spherically symmetric solution $\phi^c = \log \frac{2}{\mu^2 r^2}$, which is invariant under scale transformations.

The next step is to analyse this theory with the renormalization group. This non-perturbative approach is the most powerful way of deriving scaling behaviours in field theory (for example refs 4, 9, 11). The correlation length ξ , for infinite-volume systems, becomes infinite at criticality, because $\xi \sim \Lambda^{-\nu}$ as $\Lambda \rightarrow 0$; $\Lambda = \frac{\mu^2}{T_{\text{eff}}}$ is the distance to the critical point ($\Lambda = T - T_c$ in condensed-matter and spin systems) and varies according to the renormalization group transformations. As our system is critical at a finite size R , it is not singular, and we have $\xi \sim R$, $\Lambda \sim R^{-1}$. The mass density $m\rho \sim e^\phi$ is identified with the energy density of the renormalization group (also called thermal operator). The partition function can be written as

$$\mathcal{Z}(\Lambda) = \iint \mathcal{D}\phi e^{-S^* + \Lambda \int d^2x \phi(x)} \quad (6)$$

where S^* is the action at the critical point. As the theory of the scalar field has a scaling behaviour as seen in the perturbative approach (that is, it is critical), we can write $\log \mathcal{Z}$ as the sum of a power-law function of Λ and an analytical function $F(\Lambda)$, such that

$$\frac{1}{V} \log \mathcal{Z}(\Lambda) = \frac{K}{(2-\alpha)(1-\alpha)} \Lambda^{2-\alpha} + F(\Lambda) \quad (7)$$

where $V \sim R^3$ is the volume, K is a constant and α is the thermal critical exponent.

Calculating the second derivative of $\log \mathcal{Z}(\Lambda)$ with respect to Λ (at constant V) from equations (6) and (7) and equating the results, gives

$$\frac{\partial^2}{\partial \Lambda^2} \log \mathcal{Z}(\Lambda) \approx KV \Lambda^{-\alpha} \approx R^{2/\nu} \quad (8)$$

where we used the fact that $\Lambda \sim R^{-1/\nu}$ and the scaling relation $\alpha = 2 - 3\nu$ (ref. 9). The right-hand-side of equation (8) gives the mass fluctuations squared $(\Delta M(R))^2 \equiv \langle M^2 \rangle - \langle M \rangle^2$. Hence,

$$\Delta M(R) \sim R^{d_H}$$

Therefore, the scaling exponent ν can be identified with the reciprocal of the Hausdorff (fractal) dimension d_H of the system

$$d_H = \frac{1}{\nu}$$

The perturbative calculation gives the mean field value for ν (ref.

11). That is,

$$\nu = \frac{1}{2}, \quad d_H = 2 \quad \text{and} \quad q = \frac{1}{2} \quad (9)$$

The renormalization group transformation however, replaces the parameter μ^2 in $S[\phi]$, by an effective parameter for the scale in question. This approach shows that the long-distance critical behaviour is governed by the (non-perturbative) Ising fixed point^{4,9}. There are, very probably, no further fixed points¹⁰. The scaling exponents associated with the Ising fixed point are

$$\nu = 0.631, \quad d_H = 1.585 \quad \text{and} \quad q = 0.293 \quad (10)$$

In the renormalization group analysis, the two-point density correlation function behaves as $r^{\frac{2}{d}-6}$ or $r^{-2.830}$ at large distances (compared to r^{-2} for mean field). This should be compared to the observations. Previous attempts to derive correlation functions from observations were not conclusive owing to the lack of dynamical range¹², but greatly extended maps of the ISM could soon be available to test our theory. We predict an independent exponent for the gravitational potential correlations ($\sim r^{-1-\eta}$, where $\eta_{\text{Ising}} = 0.037$ or $\eta_{\text{meanfield}} = 0$; ref. 9), which could be checked by gravitational lens observations in front of quasars.

The mean-field exponents describe the situation where nonlinear field fluctuations are negligible. When nonlinear fluctuations are strong, the renormalization group exactly accounts for their contributions, giving the Ising-fixed-point exponents.

If we consider the mass of the particles to be that of the neutral hydrogen atom, with $T \approx 3$ K, and we estimate the fugacity z using the ideal gas value $z = \rho_0 \left(\frac{h^2}{2\pi m k T} \right)^{3/2}$, we find the length $\mu^{-1} \approx 30$ AU (4.5×10^{14} cm), and the dimensionless coupling $g^2 \approx 5 \times 10^{-53}$, for a density $\rho_0 \approx 10^{10}$ atoms per cm^3 (ref. 8). This extremely small value for g supports the use of the perturbative method at these scales, implying the mean-field values for the exponents in equation (9). However, the effective coupling constant g grows with the scale, according to the renormalization group flow (towards the Ising fixed point); μ^{-1} is of the order of the smallest distance where the scaling regime applies, and corresponds to the smallest observed gravitational scale. Both numerical values for the critical exponents of equations (9) and (10) are compatible with the values observed in the present interstellar medium equation (2). Further theoretical work in the theory of the scalar field will determine whether the scaling behaviour is given by the mean field or by the Ising fixed point.

We have taken the simplified view that the ISM is an isothermal self-gravitating gas. This idealized system describes exactly the outer parts of galaxies, far from any star formation and heat sources. There, the molecular-cloud ensemble must be in isothermal equilibrium with the cosmic background radiation at

$T \approx 3$ K (for example refs 8, 13). Well inside the Galaxy, the physics of the ISM is much more complex, especially when the violent perturbations due to star formation are taken into account. Locally, the ISM around star formation regions can, in effect, lose its fractal structure, at least partially (it becomes diffuse and much less fragmented—it is more or less ionized). But radiative cooling is very efficient, and shock waves are highly dissipative, so that globally, at large scales, the interstellar medium can still be considered as isothermal. The bulk of the ISM is only slightly perturbed and we have shown that the scaling laws are stable under perturbations, so that we believe that our theory applies to most of the ISM (and in particular to the low star-forming Taurus region shown in Fig. 1).

Turbulence is probably relevant in the dynamics of the ISM³, but this could be a consequence of the fractal structure built up by gravitational instabilities. It has been recognized for a long time that the relationship between the size and the linewidth in the ISM is similar to the Kolmogorov law $\Delta v \propto R^{1/3}$ derived for incompressible subsonic turbulence (this assumes that the energy flow per unit mass ($\epsilon \propto \Delta v^3/R$) is constant all over the hierarchy, and is finally dissipated on the smallest scales by viscous processes). The energy would be supplied at large scales by galactic rotation¹⁴. Gravitationally driven, compressible turbulence¹⁵, as well as gravitational clouds in quasistatic virial equilibrium¹⁶ give the mean-field exponents of equation (9).

The important point is that self-gravity alone can account for the fractal structure of the ISM, and quantitatively predicts its fractal dimension and related critical exponents. A new and

unexpected connection between the ISM and critical phenomena is revealed. It is interesting to note that the gravitational gas has been found at critical conditions, with correlations at all scales, and with scale-independent, power-law relations for a continuous range of physical parameters (temperature, coupling constant), while the spin models with which we have found an analogy, are critical only at a single value of the temperature. This feature is connected with the scale-invariant character of the newtonian force and its infinite range. A further step in the study of the ISM would be to include the dynamical (time-dependent) description within the field-theory approach which we have presented. $\square$

Received 3 June; accepted 30 July 1996.

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A polymeric optical pattern-recognition system for security verification

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POLYMERS that exhibit the photorefractive effect—a light-induced modulation of refractive index—are emerging as attractive materials for optical devices and processing systems^{1,2}. Here we demonstrate one such application using our recently developed high-efficiency photorefractive polymer². The polymer provides a nonlinear medium in which real-time all-optical image correlation, and hence pattern recognition, can be accomplished. This forms the basis of an optical security system, whereby documents are encoded with practically invisible phase masks (such masks are difficult to forge), which may then be rapidly screened to verify the authenticity of the documents. The wavelengths at which our optical system operates are compatible with commercial low-power semiconductor laser diodes, and the system can be integrated into a compact device at low cost.

Rapid technological progress, especially in computers, CCD (charge-coupled device) technology, and colour printers and scanners, makes the forgery and counterfeit of valuable documents such as credit cards or other important objects, increasingly simple. Current techniques such as the embossed hologram on credit cards are no longer a reliable solution to this problem, as

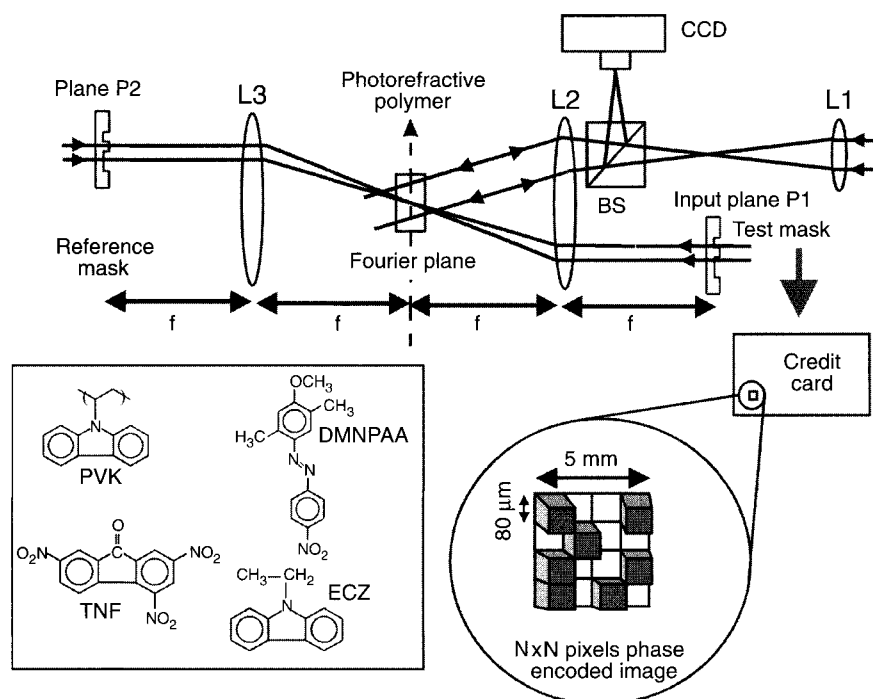
they can be copied. There is therefore a need to develop new and inexpensive optical methods for security applications³ to handle the counterfeiting problem better. Optical security features can be inspected by either visual checking without special equipment or with the help of technical facilities for rapid screening. When such an optical system is required for security checking, low manufacturing cost is a critical issue for its viability.

The low-cost security verification system we propose here is based on the optical encoding of documents with pseudo-randomly generated phase masks⁴ (these are optical elements that encode information in the phase, rather than the amplitude, of transmitted or reflected light). The inspection of these masks is performed by obtaining the all-optical spatial correlation of two phase-encoded images in a photorefractive polymer in a four-wave mixing configuration (Fig. 1). One phase mask is placed on the object to be verified, for example a credit card. The other is made available to the security systems for comparison with the input image. The phase-encoded images may include a combination of biometric information, for verification of the individual carrying the card, and a secret code known to the system for authentication of the card. A phase mask is practically invisible and may be permanently attached to the object to be verified. It can be manufactured by a number of techniques including embossing on plastic films and encoding on photopolymer. With the high resolution of commercially available optical materials, the phase mask can be of the order of a million pixels and the mask size will be only a few square millimetres.

Pseudo-random codes or pseudo-noise sequences can be employed to encode the phase masks⁵. These codes have unique correlation properties. Their autocorrelation is δ -function-like (as in the autocorrelation of white noise) with small sidelobes and with a maximum correlation value proportional to the number of the pixels, N , in the code. The average cross-correlation between two different codes with the same number of pixels is $1/N$ times the peak value of the autocorrelation which results in an average discriminating power of N . Using optical materials, two-dimensional optical codes with large numbers of pixels (that is, large N)

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FIG. 1 Schematic of the four-wave mixing photorefractive correlator for security applications. L2 and L3 are the Fourier transform lenses (focal length $f = 85$ mm). The beams overlap in the back focal plane of the lens L2 and form an interference fringe pattern. A photorefractive polymer is positioned in the region of overlap and a phase grating is formed in it by a photorefractive process duplicating the incident light intensity pattern. The signal emerges counterpropagating with the second writing beam. The diffraction occurs on the regions of the hologram where the Fourier transforms of the two masks overlap. Thus the recorded hologram of the Fourier-transformed test mask acts as a complex filter for the read-out beam bearing information about the master mask. BS is a beam-splitter. CCD is a camera. The inset shows the chemical structure of the components of the photorefractive polymer used in the experiments.



can be employed as phase masks. This results in small cross-correlation outputs for any two different codes. Consequently, the autocorrelation properties of large pseudo-random codes enable confident verification of authorized codes, while their cross-correlation properties enable confident discrimination against unauthorized codes. The codes are also robust to degradation of the individual pixels owing to their correlation properties. If m pixels of a code are degraded, we expect the peak-to-sidelobe ratio to decrease by an average of m/N compared with the original code. If for instance 90% of the surface of the mask is damaged, the normalized autocorrelation signal will decrease from 1 to 0.1. As cross-correlations will still average at $1/N$, the average discriminating power will be $0.1 \times N$. For 64×64 phase masks ($N = 4,096$), the 0.1 autocorrelation signal of a 90% damaged mask will still be 41 times higher than the signal of a (rarely) high cross-correlation value of $10/N$.

Research in this area of optics has been stimulated by the potential for parallel information processing. However, a combination of the limited performance and the high cost of existing nonlinear optical materials has severely limited the application of all-optical correlators. Inorganic photorefractive crystals have been investigated⁶ but their processing and high cost has limited their widespread use. To improve processing conditions, polymers with third-order susceptibility $\chi^{(3)}$ have also been proposed in the past^{7,8}. However, their low efficiency requires very high peak power laser pulses that can only be generated by sophisticated ultra-short pulse laser sources which makes them impractical. Owing to limitations of previously available optical materials, other correlator designs have been proposed. For instance, nonlinear joint-transform correlators show good performance for pattern recognition and are capable of real-time operation⁹. However as these systems use sophisticated liquid-crystal light valves¹⁰ or CCD detectors, and perhaps a computer to perform Fourier transforms, they do not meet the low-cost requirement.

The proposed optical correlation system is illustrated in Fig. 1. The input card containing the phase mask is placed in the plane P1 which is to be correlated with the master phase image in the plane P2. The hologram, written by the interference of the reference beam and the beam going through the test mask, forms a holographic filter for the master mask. Recording of this holographic filter was performed in real time with a 633-nm He-Ne laser with a

total power of 1.5 mW. A separate 675-nm laser diode with <1 mW power was used for the read-out. The spatial cross-correlation function of the two phase masks was formed in the plane P1. The illuminated area on the sample was about 4 mm in diameter at normal incidence. As in regular four-wave mixing experiments, the sample was tilted at 60° . Writing beams were s-polarized (perpendicular to the plane of incidence defined by the sample-normal and the wavevector of the incident light, that is vertical in this case), and the read-out beam was p-polarized (horizontal). The diffracted beam was picked up by the beam-splitter BS and projected onto a CCD camera to record the correlation. Here we used a CCD camera only to visualize the correlation peak and to compare it with the background noise. In practice the correlation can be verified with a standard silicon detector.

The active medium was a 105-μm-thick photorefractive polymer film sandwiched between two transparent indium-tin-oxide electrodes. For this demonstration, we used the composite DMNPAA:PVK:ECZ:TNF (see Fig. 1 inset) with the composition 50:37:12:1 wt% (ref. 2). A field of $50 \text{ V } \mu\text{m}^{-1}$ was applied during the recording of the holographic filter and the implementation of the autocorrelation. Due to its low glass transition temperature ($T_g = 16^\circ\text{C}$), this photorefractive polymer can record and retrieve optical information only when an electric field is applied. The applied field increases the photogeneration efficiency and is the drift force for the transport of the photo-carriers; it is also responsible, together with the internal space-charge field, for the orientation of the nonlinear optical molecules. The resulting photorefractive refractive index changes, through electro-optic and orientational effects, are the basis of the optical recording of the holograms. The very low current (a few nanoamperes) that flows through the device makes the power consumption negligible. The polymer composite we used has an average lifetime of six months at room temperature, when encapsulated and protected from moisture owing to its high chromophore concentration (50 wt%). Chromophore crystallization and phase separation are responsible for the eventual failure of the photorefractive polymer. We have recently enhanced the lifetime of our polymers¹¹ either by reducing the chromophore concentration or by using eutectic mixes of different chromophores. Such samples have a diffraction efficiency that is lower than that of the

sample used here, but still show a diffraction efficiency (a few tens of a per cent) that is sufficient for this type of correlator. The stability problem can be completely resolved by synthesizing low- T_g side-chain functionalized photorefractive polymers. The stability of several years which can be obtained from such materials should by no means be considered a limitation to the practical applicability of the proposed system.

For the purpose of this demonstration experiment, the phase masks were binary, random patterns of 64×64 pixels; the whole array size was 5×5 mm, and was formed in a 2- μ m-thick photoresist layer with a refractive index $n = 1.64$ deposited on a glass substrate corresponding to a phase-modulation depth of 4π . In principle, larger codes and grey-level patterns can be used to improve the discriminating power. The master phase patterns can easily be compared in a phase-only spatial light modulator in real time, which allows scanning of the database of control masks without changing them.

Figure 2 shows an example of the cross-correlation of the master phase mask with a matching phase mask. It clearly demonstrates a sharp correlation peak in the centre which signifies a match. A mask different from the control image produces only the randomized background, shown around the base of the peak in Fig. 2. Therefore, discrimination between the original and any copy is achieved by measuring the light intensity in the centre of the correlation plane and comparing it with a threshold that is adjusted for a chosen security level.

The security verification system we propose has the following features that make it practical for widespread applications. First, the use of a highly efficient photorefractive polymer as active material in an all-optical correlator configuration and its compatibility with semiconductor laser diodes keep the overall manufacturing cost to levels that are significantly lower than that of any previously proposed optical correlator. Second, the system is fast because the processing is implemented optically in parallel. Third, the high resolution of the photorefractive polymers allows the use of shorter-focal-length lenses in the $4f$ correlator (where f is the focal length of the Fourier transform lenses), thus making its design more compact compared with one using liquid-crystal light valves. Fourth, all the components (including the laser source and the nonlinear material) can be manufactured in a very small size and the system can easily be further miniaturized. Last, as the recording process is based on the photorefractive effect, the stored hologram can be erased and a new hologram can be written in real time. This reversible real-time recording and processing enables the testing of a variety of different documents encoded

with different phase masks, and their comparison with a corresponding master mask database. Its response time (~ 1 s) is satisfactory for the security verification tasks and does not preclude fast database searches. As explained above, the use of pseudo-random codes ensures that the system is robust and resistant to noise acquired during wear.

Phase encoding can be added to biometric identification features such as fingerprints or pictures for additional security. The larger the number of available techniques, the better the changes of preventing credit card (and document) fraud.

We believe that the security verification system presented here has a number of important technical advantages over previous designs that greatly increase its potential for use in numerous security applications. This potential is further enhanced by the recent emergence of technologies for producing very low cost precision, plastic optical elements such as lenses and splitters by precision injection moulding. $\square$

Received 13 February; accepted 11 July 1996.

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ACKNOWLEDGEMENTS. This work was supported by the US office of Naval Research through the Center for Advanced Multifunctional Nonlinear Optical Polymers and Molecular Assemblies (CAMP), the US National Science Foundation, by the USAF Office of Scientific Research, and by the US Rome Laboratory, Hanscom Air Force Base.

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Off-the-shelf proteins that rival tailor-made antibodies as catalysts

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MIMICKING the efficiency of enzyme catalysis is a daunting challenge. An enzyme selectively binds and stabilizes the transition state(s) for a particular reaction^{1,2}. Artificial host systems can bind ground states just as efficiently³, and rate enhancements comparable to those in enzymatic reactions can be achieved by bringing catalytic and substrate groups together in intramolecular reactions. But the combination of selective binding and efficient catalysis remains elusive. The best enzyme mimics currently known are catalytic antibodies^{5,6}. They bind transition-state analogues with high affinity, but their catalytic efficiency generally falls far short of that of enzymes^{4,8}. Thorn *et al.*⁹ recently described an antibody that catalyses the eliminative ring-opening of a benzisoxazole “exceptionally efficiently” using carboxylate as the general base, raising the intriguing possibility that this high efficiency derives from precise positioning of catalytic and substrate groups¹⁰. Here we show that familiar ‘off-the-shelf’ proteins—serum albumins—catalyse the same reaction at similar rates, using a lysine side-chain amino group as the catalytic general base. Comparisons suggest that formal general base catalysis is of only modest efficiency in both systems, and that the antibody catalysis is boosted by a non-specific medium effect.

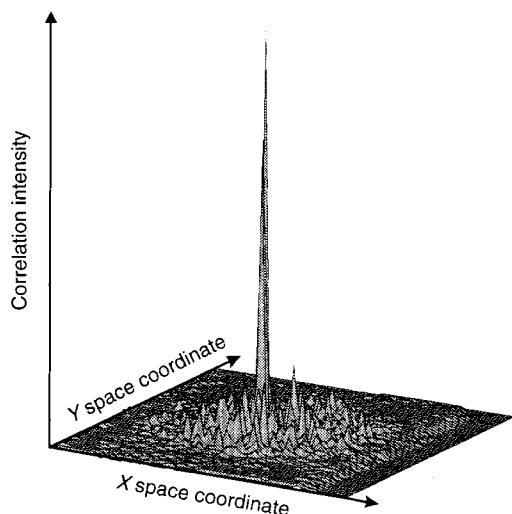


FIG. 2 Spatial light intensity distribution in the correlation plane P1 (see Fig. 1) with a test phase mask matching the master mask. The image in the plane P1 was recorded by a CCD camera.

The reaction shown in Fig. 1 (conversion of molecule **1** → **2**) is well understood¹¹: it involves the simple one-step mechanism shown, in which removal of the proton from carbon by a general base B is concerted with N–O bond cleavage. Proton transfers are

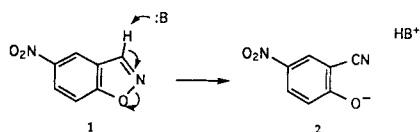


FIG. 1 Mechanism of the Kemp elimination.

involved in almost every enzyme reaction. They can become rate-determining, and thus need effective catalysis, especially when the proton is transferred to or from carbon, or when the reaction is concerted with the formation or cleavage of a bond between heavy (that is, non-hydrogen) atoms. But proton transfer in intramolecular model reactions is almost always exceptionally inefficient, general acid or general base catalysis being characterized by low effective molarities (EM: the nominal concentration of an intermolecular catalytic group needed to match the rate of the intramolecular reaction) of the order of 1–10 M (ref. 12). We may assume that enzyme-catalysed proton transfers are not inefficient¹³, so the models must be deficient. A likely explanation has been identified: where the transition state for the proton transfer is stabilized by strong intramolecular hydrogen bonding, EMs of up to 10^6 M can be achieved^{14–17}. A key factor is the very precise positioning of the catalytic group. This can be achieved in intramolecular reactions by careful design, and is fundamental to the arrangements at an enzyme active site. But antibody catalysis does not usually involve a catalytic group, still less one that is precisely positioned, so the evidence of Thorn *et al.*⁹ that the reaction catalysed by antibody 34E4 involves a general base in the binding site, acting with an EM as high as 4.1×10^4 M, deserves careful appraisal¹⁰.

The general base in the reaction catalysed by antibody 34E4 was identified as carboxylate. As originally pointed out⁹, the apparent EM is undoubtedly due in part to activation by desolvation of this carboxylate group in the hydrophobic environment of the antibody–substrate complex. Comparable rate accelerations are observed for the acetate-catalysed conversion of **1** to **2** when the solvent is changed from water to a dipolar aprotic solvent such as acetonitrile¹⁸. So it is possible that the contribution to transition-state stabilization of the precise positioning of the carboxylate general base is a small factor, with the major part of the catalytic power of antibody 34E4 arising from this medium effect.

This background prompted us to look for catalytic activity in

TABLE 1 Kinetic parameters for reactions of **1** catalysed by BSA and HSA

Catalyst	pH	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/k_{uncat}
BSA	6.8	1.37 ± 0.12	0.71 ± 0.04	ND
BSA	8.0	2.07 ± 0.49	4.7 ± 1.1	4×10^3
BSA	9.0	0.69 ± 0.10	14.6 ± 1.23	2.5×10^3
HSA	8.0	1.55 ± 0.49	3.2 ± 0.73	2.7×10^3
HSA	9.0	3.06 ± 1.2	28.8 ± 9.7	5×10^3

Five different commercial samples of BSA (purified by different methods) were assayed at different pHs and substrate concentrations; all had similar specific activities. This is a strong indication that the observed catalytic activity is not due an enzyme contaminant. The low solubility of **1** in water prevented measurements at substrate concentrations above K_m ; hence even larger errors in K_m , and thus in k_{cat} , than those indicated cannot be excluded. Experimental conditions are described in Fig. 2 legend. ND, not determined.

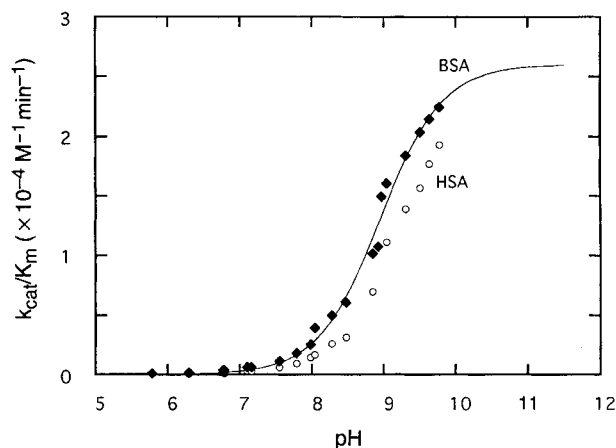
other proteins, known to have hydrophobic binding sites with general base residues in close proximity. We report a group of ‘off-the-shelf’ protein catalysts—the serum albumins—which rival the catalytic antibodies of Thorn *et al.*⁹ in activity, but use a lysine side chain as the catalytic general base, thus allowing an assessment of the contribution of the medium effect to catalysis. (The amine-catalysed Kemp elimination is relatively insensitive to medium effects, as discussed below.)

We find that the eliminative ring-opening of **1** to form **2** is catalysed by bovine serum albumin, and by serum albumins from other species (consistent with the high sequence homology of serum albumins¹⁹—and the even higher similarity expected for their three-dimensional structures, as demonstrated for human and horse serum albumin^{19,20}). Kinetic parameters for the reactions catalysed by bovine and human serum albumin (BSA and HSA) are shown in Table 1.

Catalysis by both BSA and HSA proceeds with multiple turnover, though after more than 10 turnovers the rate gradually decreases to the background rate. This is the result of product inhibition (K_i is estimated to be 50 μM , compared with $K_m = 0.7$ – 3.1 mM for the uncharged substrate **1**), rather than inactivation of the protein: full activity of both albumins is restored after dialysis against reaction buffer. This inhibition by the product anion (**2** has a pK_a of 4.1; ref. 11) is consistent with the presence of positively charged active-site residues (antibody 34E4, with a carboxylate anion as the adjacent general base, does not suffer from product inhibition); and analysis of the pH dependence of the albumin-catalysed reactions suggests the participation in their basic form of side chains with pK_a values of 8.95 for BSA and 9.17 for HSA

FIG. 2 Plot of k_{cat}/K_m for the HSA- (circles) and BSA-catalysed reaction **1** → **2**, as a function of pH. The curve drawn for the BSA-catalysed reaction is calculated using $pK_a = 8.95$ and $(k_{cat}/K_m)_H = 2.6 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$. The HSA data could be fitted similarly using $pK_a = 9.17$ and $(k_{cat}/K_m)_H = 2.37 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$. K_m varies little with pH (Table 1), so that differences in the rates at different pHs are primarily due to changes in k_{cat} . At pH > 10, accurate rate constants could not be obtained because of the high rate of the buffer-catalysed, background reaction at these pHs.

METHODS. Rates were determined by monitoring the release of **2** at 405 nm in a microtitre plate reader. Initial velocities were determined at $25 \pm 1^\circ\text{C}$ with **1** (0.15–0.8 mM) and serum albumin (15 μM), and are corrected for the rate of the background reaction under the same conditions. Data points each represent three separate measurements. Kinetic parameters were derived by the Lineweaver–Burk and Eadie–Hofstee methods, or by direct analysis²⁹. Errors are calculated, based on standard deviations in k_{cat} and K_m values obtained from independent measurements and the different methods. k_{uncat} was determined as a function of buffer concentration at pH 8.0 and 9.0 at $25 \pm 1^\circ\text{C}$ by the method of initial rates and extrapolated to zero buffer concentration. The values obtained, $1.18 \times 10^{-3} \text{ min}^{-1}$ and $5.75 \times 10^{-3} \text{ min}^{-1}$, for pH 8.0 and 9.0 respectively, compare well with the data collected by Kemp *et al.* at 30°C (ref. 18) for the same substrate. pH–rate measurements were made under pseudo-first-order conditions ($S_0 \leq 0.15 \text{ mM}$). Buffers (30 mM, con-



taining 150 mM NaCl) were: for pH 5.7–6.8, bis-Tris; for pH 6.8–7.8, HEPES; for pH 8–9.0, bicine; for pH 8.5–9.7, AMPSO; for pH 9.5–10, AMP. (Buffers containing hydrophobic residues, such as cyclohexyl groups, inhibit the reaction.)

(Fig. 2). This and further evidence described below suggest that the general bases involved are the ϵ -amino groups of lysine side chains. Crystal structure and related studies indicate a cluster of positively charged lysine and arginine residues in HSA and in other albumins, which interact with anionic ligands¹⁹. Catalysis is also inhibited by a series of anionic ligands, including octanoate and drugs such as Ibuprofen, Warfarin and Naproxen, which bind specifically to HSA, indicating that catalysis occurs primarily at two specific, previously identified sites²¹.

The active involvement of lysine residues is consistent with the observed inhibition of catalytic activity by side-chain-specific covalent modification. Pyridoxal phosphate forms a covalent complex with bovine serum albumin, the aldehyde group reacting with one or two adjacent lysine side chains to form a Schiff base or a gem-diamino group. Two equivalents of pyridoxal phosphate react with BSA at two distinct sites, with different affinities^{22,23}. Up to 80% of the catalytic activity of BSA towards **1** is inhibited by incubation with two (or more) equivalents of pyridoxal phosphate. Pyridoxal phosphate reacts less favourably with HSA^{22,23} and the catalytic activity of HSA is correspondingly less sensitive to added pyridoxal phosphate (up to 40% of activity lost). These modifications affect the basicity of the lysine amino groups and may interfere with substrate binding.

We now have enough information for an informed comparison of catalysis of the Kemp elimination by serum albumins and by catalytic antibodies^{9,24}. Turnover numbers at the respective pH optima are comparable: k_{cat} for catalytic antibody 34E4 is 39.6 min^{-1} at pH 7.4, where activity is maximal⁹, whereas k_{cat} for HSA is 71 min^{-1} near pH 10. (Because maximum rates are reached for serum albumins at higher pH values, the rate acceleration ($k_{\text{cat}}/k_{\text{uncat}}$) in water at pH 7.4 is ~ 100 -fold higher for antibody 34E4.) Thus the two classes of protein catalyse the conversion of **1** to **2** with similar efficiency, with the benzisoxazole ring bound at a hydrophobic site in each case, but use different catalytic general bases, and have different pH optima. Because primary amines are more strongly basic, and thus intrinsically more reactive as general bases than carboxylate anions, we use EMs for the comparisons that follow.

The reactivity as a general base in the Kemp elimination of a carboxylate anion like acetate is much higher (over 10^7 times) in acetonitrile than in water, whereas the reactivity of an amine is rather insensitive to the medium (Table 2). This reflects the strong solvation of carboxylate anions in hydrogen-bond-donor solvents. Such stabilizing solvation is not available in polar aprotic solvents like acetonitrile, which however solvate amines and alkylammonium cations reasonably well. Comparison of the rate of the HSA-catalysed reaction with the rate constant for the reaction catalysed by the primary amine in water and acetonitrile gives similar effective molarities, with $k_{\text{cat}}/k_{\text{amine}}$ being 28 and 3.2M, respectively. But for the antibody-catalysed reaction the EM is high only if acetate (AcO^-) in water is used as the reference ($k_{\text{cat}}/k_{\text{OAc}} = 4.1 \times 10^4 \text{ M}$); if (to take the extreme case) k_{OAc} in acetonitrile is used for comparison, the resulting EM ($2.3 \times 10^{-4} \text{ M}$) is much smaller than the values observed with the serum albumins (Table 2). The 'correct' comparison will give an EM that lies between these two extremes, and there is no reason to suppose that this will be significantly higher than those for the albumin-catalysed reactions. We conclude that most of the 'remarkable efficiency'⁹ of the catalytic antibodies does in fact result from activation of carboxylate by the apolar environment of the active-site (a property mimicked by the acetonitrile solvent), and that the contribution to the EM of the carboxylate group of

TABLE 2 Dependence on medium of amine- and carboxylate-catalysed reactions of **1** and effects on estimates of catalytic efficiency

General base, medium	Catalysis by simple general bases		Catalysis by proteins		
	k_2 ($\text{dm}^3 \text{ mol}^{-1} \text{ min}^{-1}$)	$k_2(\text{CH}_3\text{CN})$ $k_2(\text{water})$	EM ($= k_{\text{cat}}/k_2$)/M*		
			Antibody 34E4†	BSA	HSA
CH_3COO^- in water‡	8.4×10^{-3}	2×10^7	4.1×10^4		
in CH_3CN	1.69×10^5		2.3×10^{-4}		
$\text{CH}_3\text{OCH}_2\text{CH}_2\text{NH}_2$ in water§	1.02	8.5		14	28
in CH_3CN §	9.0			1.6	3.2

* EM based on k_2 (column 2).

† Values taken from ref. 9.

‡ Data from ref. 18 for **1** at 30 °C.

§ Rate constants were measured with **1** at 25 °C with 2-methoxyethylamine (pH at 50% free base equals the pK_a of 9.75).

|| A similar ratio was observed by Kemp *et al.*¹⁸ for triethylamine.

the antibody from the precise positioning of the active site carboxylate side chain is modest—that is, normal for general base catalysis¹².

There is a general lesson to be learned from these results. Our ability to produce effective enzyme mimics is in almost all cases limited to what can be described as 'model reactions'²⁵. These are reactions characterized by a high thermodynamic driving force, a low enthalpy of activation (for example, $\Delta H^\ddagger$ for the conversion of **1** to **2** in water is $-39 \text{ kcal mol}^{-1}$ and $\Delta H^\ddagger$ is $\sim 12 \text{ kcal mol}^{-1}$; ref. 11) and a single rate-determining transition state (as identified for the Kemp elimination¹¹). Serum albumins are known to catalyse other 'model reactions', including the hydrolysis of activated esters like 4-nitrophenyl acetate²⁶, and the decomposition of a Meisenheimer complex (also catalysed by a lysine ϵ -amino side chain, with a pK_a of 8.4, acting as a general base^{27,28}). As observed for the Kemp elimination, turnover numbers (k_{cat}) and rate accelerations ($k_{\text{cat}}/k_{\text{uncat}}$) observed with serum albumins are not very different from those observed with most antibodies catalysing similar reactions⁴. This reflects the relatively simple requirements of an active site catalysing this type of reaction: the fortuitous combination of hydrophobic cavities and reactive side chains available in serum albumins being sufficient in favourable cases. (Catalysis based on substrate binding is by no means a general, or even common property of proteins with functionalized hydrophobic cavities: potential catalytic groups must still be within range.)

Most reactions catalysed by enzymes are of course not 'model reactions', but demanding transformations with high activation energies¹³, complex mechanisms and several consecutive transition states and intermediates. The requirements of the catalyst are stringent, each step demanding precise positioning of active-site catalytic groups and of the substrate, in a specifically structured environment. This is where most known enzyme mimics—and certainly randomly selected protein catalysts such as the serum albumins—fail. Catalytic antibodies elicited against transition state analogues are the most effective enzyme mimics currently available, but they are not—as yet—capable of efficient catalysis of demanding reactions of the type handled so effectively by enzymes.

Received 29 April; accepted 18 July 1996.

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ACKNOWLEDGEMENTS. We thank D. Hilvert for his constructive comments. F.H. also thanks St John's College for a studentship and the Technical University of Berlin for an Erwin-Stephan Prize. D.S.T. is grateful to FEBS for a fellowship, and thanks A. Fersht for support. A.J.K. is coordinator of ENABC (European Network on Antibody Catalysis).

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Chlorin accumulation rate as a proxy for Quaternary marine primary productivity

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A KNOWLEDGE of past changes in the biological productivity of the oceans is important for understanding the interactions between carbon cycling and climate. Phytoplankton productivity in today's oceans can be estimated from the concentrations of chlorophyll in sea water¹, but chlorophyll is not preserved in the sediments. Existing proxies for past algal productivity do not represent total productivity; for example, biogenic opal² reflects the contribution of only part of the phytoplankton community, and the organic carbon record can be subject to contamination from terrestrial inputs^{2,3}. Although chlorins, the pigment-transformation products of chlorophyll, are widespread in Quaternary marine sediments, their potential as proxy measures of past variations in primary productivity has not been convincingly demonstrated. Here we report a high-resolution molecular stratigraphic record of chlorin concentrations over the past 350,000 years in a sediment core from the subtropical Atlantic continental margin. Maxima in the chlorin accumulation rate coincide with significant peaks in the accumulation rates of biogenic opal (at the end of glacial terminations) and organic carbon (between terminations). These results suggest that chlorins, unlike other proxies, can serve as a measure of total primary productivity variations.

Site 658 (holes A, B and C; Ocean Drilling Program leg 108; 20° 44.95' N, 18° 34.85' W) was cored off Cap Blanc, northwest Africa (Fig. 1) in 2,270 m water depth⁴. The site was chosen because it lies on the outer edge of a promontory projecting

from the continental slope between two canyons, so it is thought to be protected from gravity-driven downslope sediment transport^{4,5}. The high-resolution seismic stratigraphy suggests an undisturbed sedimentary record⁴, and the present day setting produces permanent, trade-wind-induced upwelling of nutrient-rich waters, which leads to a west African maximum in productivity (>500 g m⁻² yr⁻¹)⁶ and results in high and essentially linear average sedimentation rates (estimated at 15–20 cm kyr⁻¹) and organic carbon (C_{org}) contents of up to 3% (ref. 4). Cores from the site have previously been studied to investigate the record of variations in palaeoproductivity (from C_{org} accumulation rate⁷) and the molecular sea surface temperature (SST) record on ~100-year timescales over the past 350 kyr (ref. 8).

Solvent extracts of the sediments contain a variety of chlorins derived from phytoplanktonic chlorophyll *a* (P.G.H. *et al.*, unpublished results), whose total concentrations have been measured using fluorescence spectrophotometry⁷. From comparison with accepted proxies of palaeoproductivity, that is, biogenic opal concentration and percentage C_{org} (ref. 10) (Fig. 2; shown on a composite depth scale in metres below sea floor, m.b.s.f.), it appears that the chlorin concentration also results from variations in primary productivity, which are mainly controlled by the availability of nutrients in the surface water.

The biogenic opal accumulation rate (g m⁻² yr⁻¹), derived from the concentration data, generally varies from ~0.5 to 1.5 between 0 and 150 kyr, and from about 1.0 to 2.8 further down-core (Fig. 3c). However, there are distinct maxima at the end of the last four glacial terminations, which decrease from 60 to 2.5 for terminations IV to I, respectively, and which typify the early interglacial substages 9.3, 7.53, 5.53 and 1.1 (Fig. 3e). The gap in the δ¹⁸O record (Fig. 3e) between 50 and 85 kyr, although previously attributed to a hiatus¹¹ in hole 658B, has since been reinterpreted¹² as a drilling artefact, by reference to contemporaneous sections of hole 658C (ref. 12), probably caused by disturbance during coring. We show, however, the chlorin and C_{org} accumulation rates for hole 658C (Fig. 3) over this age range to provide a comparison between the two, although we recognize that there may be some inaccuracy in dating samples in this region. These maxima in the opal record coincide with precessional summer insolation maxima (Fig. 3d) at 333, 241, 126 and 10 kyr, respectively, within the stratigraphic error range of ~2,000 years (ref. 5). They also coincide with maxima in the chlorin accumulation rate (Fig. 3a), which are as high as 200 µg cm⁻² kyr⁻¹, except near termination III, where an 80-cm coring gap disturbs the record and makes the

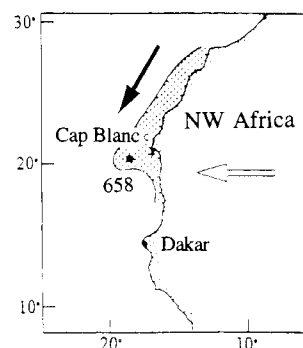
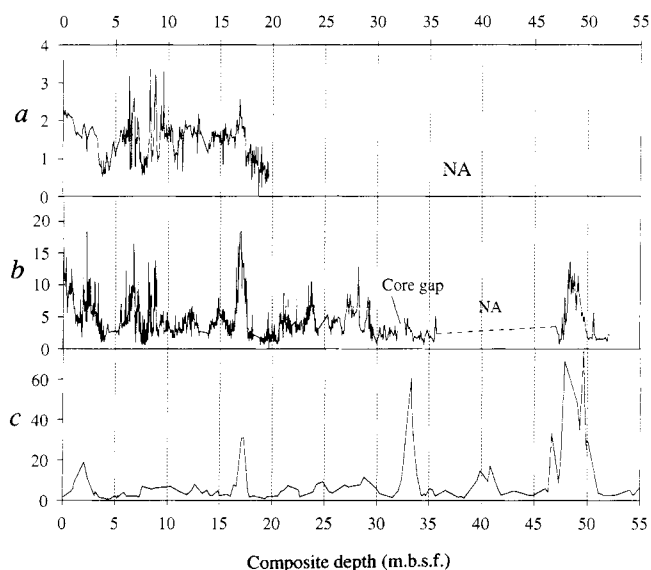


FIG. 1 Location of ODP leg 108, site 658 (20° 44.95' N, 18° 34.85' W) off northwest Africa (modified from ref. 28). Pre-site surveys of bottom topography⁴ indicate that the site provides a largely undisturbed palaeo-environmental record; it lies directly beneath a near-shore area of permanent upwelling (shaded area for Pliocene/Pleistocene) in the central region of dust supply from northeast trade winds (black arrow) which control upwelling intensity, and below the dust supply of the Sahara air layer (white arrow). Cores from hole 658C were deep-frozen immediately upon recovery as a precaution to maintain organic integrity before analysis.

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FIG. 2 Site 658 record (NA, not analysed) on a composite depth scale (metres below sea floor, m.b.s.f.) of: a, C_{org} (per cent; hole 658C); b, chlorin concentration ($\mu\text{g per g dry weight of sediment}$; hole 658C); c, biogenic opal (per cent; holes 658A and B; 2–63 μm fraction)¹⁰. Chlorin concentrations were determined at sampling intervals of 2 cm (corresponding on average to 130–140 yr) between ~0 to 25 kyr (0–5.0 m.b.s.f.), 4 cm between 25 and 40 kyr (5.0–7.4 m.b.s.f.), 2 cm between 40 and 180 kyr (7.4–23.4 m.b.s.f.) and 10 cm below this (23.4–35.7; 47.1–51.7 m.b.s.f.). There was an artificial coring gap of ~80 cm at ~230 kyr owing to extreme gas pressure in the sediment at the time of drilling. Extraction of freeze-dried samples (~1 g) by successive sonication and centrifugation in 10% aqueous acetone and acetone, respectively, yielded extracts that were evaporated to dryness under a stream of dry, oxygen-free nitrogen. Standard solutions of the extracts in methanol were analysed using off-column high-performance liquid chromatographic conditions, eluting with methanol¹⁹. Chlorin concentration was calculated by peak area integration and comparison with standards. The high-resolution composite depth profile was obtained for hole 658C using the magnetic susceptibility records for cross-correlation with holes 658A and B¹². This enabled matching of the proxy data from the holes.



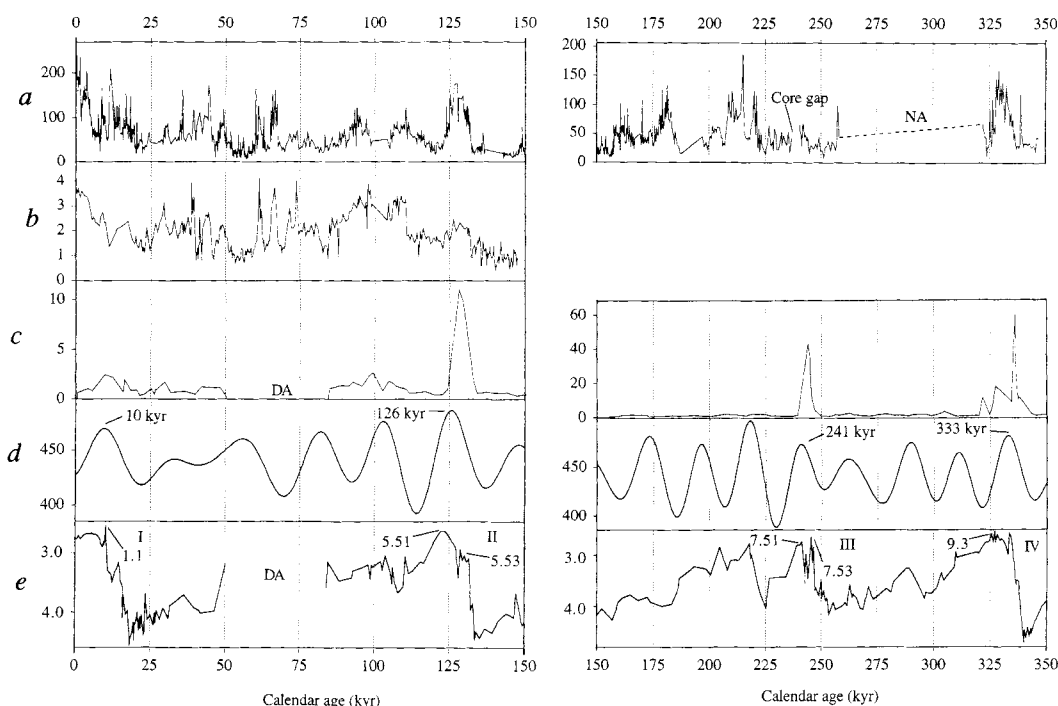
chronology less secure. There is, however, a chlorin maximum corresponding to stage 7.3, a termination-type event (near termination III) coinciding with an extreme insolation maximum.

The main influence on biogenic opal accumulation rate is diatom productivity^{13,14}, which is controlled largely by nutrient availability, especially silica, as well as phosphate and nitrate. Hence, the maxima in chlorin accumulation rate during the early interglacial stages at the end of terminations over the past 350 kyr presumably also result from increased primary productivity. The possibility that changes in the chlorin record are brought about by variation in bottom-water oxygenity seems unlikely at this site, given the magnitude of the variation^{2,15}. Furthermore, the east Atlantic has always been well oxygenated at this water depth during glacial

and interglacial stages¹⁶, except when sudden meltwater events¹⁷ brought about low oxygen phases^{16,18}. The effects of these events, recognizable as temperature lows in the molecular SST record¹², are not apparent in the rate of chlorin accumulation at the site. It also seems unlikely that the opal or chlorin maxima result from changes in any sedimentary processes because of the hemi-pelagic and largely undisturbed nature of the sequence.

The accumulation rate of chlorins indicates that productivity has varied markedly over glacial/interglacial cycles. The oceanographic setting of the site suggests that nutrients may have been brought into the area by a number of mechanisms that influenced total productivity or enhanced contributions from specific producers at certain times. For instance, the high rate of chlorin

FIG. 3 Site 658 time-series records (NA, not analysed; DA, drilling artefact) on an astronomical timescale. a, Chlorin accumulation rate (AR) ($\mu\text{g cm}^{-2} \text{ kyr}^{-1}$; hole 658C); b, organic carbon (C_{org}) AR ($\text{g m}^{-2} \text{ yr}^{-1}$; hole 658C); c, biogenic opal AR ($\text{g m}^{-2} \text{ yr}^{-1}$; holes 658A and B, 2–63- μm fraction)¹⁰; d, summer insolation record (July; 65° N)²⁹; e, benthic oxygen isotope ($\delta^{18}\text{O}$) record from holes 658A and B based on *Cibicides wuellerstorfi* (‰) versus PDB standard¹¹. Terminations I–IV and selected isotopic events are indicated for orientation. The 658A and B stratigraphy was established by matching the composite $\delta^{18}\text{O}$ curve with those from several ¹⁴C-dated cores from the North Atlantic¹¹. The age model for the first 85 kyr for hole 658C was established by correlating the detailed $\delta^{18}\text{O}$ record (first 45 kyr) and U_{37}^k SST record with several $\delta^{18}\text{O}$ and U_{37}^k records from the North Atlantic³⁰. For the remainder of the 658C record the stratigraphy was established by matching the high-resolution magnetic susceptibility record with those for 658A and B¹².



Concentration data were converted into accumulation rates³¹ using the sedimentation rate and physical property data obtained from holes 658A and B^{10,11}.

accumulation at the end of terminations relates to high diatom productivity, as is apparent from the biogenic opal accumulation rate. Changes in upwelling intensity are likely to have been a major factor in controlling productivity changes and comparison with pollen records^{19,20} indicates that increases in upwelling intensity contributed to a number of the chlorin maxima. Similarly, the increase in late Holocene wind strength^{21,22} could account for the general increase in chlorin accumulation rate to the present. Other mechanisms that may have influenced changes in productivity are variations in oceanic circulation, fertilization due to aeolian and/or fluvial input of terrestrially derived nutrients. Some evidence that the maxima in biogenic opal and chlorin accumulation rates that occurred at the end of terminations may be linked, in part, to run-off, is provided by clay and grain size accumulation rates^{10,23} and short-lived maxima²⁴ in the abundance of *Thalassionema nitschoides*, a marine diatom that reflects increased run-off¹⁴. Further investigation is necessary to assess the relative importance of mechanisms responsible for the productivity changes.

The chlorin record (Figs 2b, 3a) also closely matches the C_{org} record (Figs 2a, 3b), providing more evidence that it is a productivity indicator and that a high proportion of the organic input at this site is of marine origin². Unlike the biogenic opal record, which derives mainly from marine diatoms²⁴, it also includes contributions from other primary producers, such as coccolithophorids and dinoflagellates, which are known to have occurred at this site^{25,26} and which also produce chlorophyll *a* as the major photosynthetic pigment. Regardless of the mechanisms involved, it seems likely that the chlorin accumulation rate reflects total productivity changes, although we do not know if the response is linear.

Within a multi-proxy approach, chlorin accumulation rate represents a convenient, though not yet fully quantitative, tool for tracing carbon transfer from surface waters to sediments in the oceans with high time resolution, although further study is needed before its application as a palaeoceanographic indicator can be fully developed. In this example, one of the most important upwelling zones on the Earth, we have shown that this transfer rate has varied by up to a factor of five. This has wider importance in terms of the global carbon budget, as the continental margins are responsible for up to 50% of such carbon transfer, but comprise less than 10% of the oceans²⁷. Hence, past changes in carbon transfer in relatively small areas of the ocean that are sensitive to short-term changes in the subtropical wind system, and which have significant implications for abrupt changes in the global greenhouse effect, can be investigated using our new chlorin proxy. □

Received 3 June 1995; accepted 19 July 1996.

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ACKNOWLEDGEMENTS. We thank the NERC, the Deutsche Forschungsgemeinschaft, the EC Environment programme, and the Royal Society for support, and the ODP for samples.

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A slow earthquake sequence on the San Andreas fault

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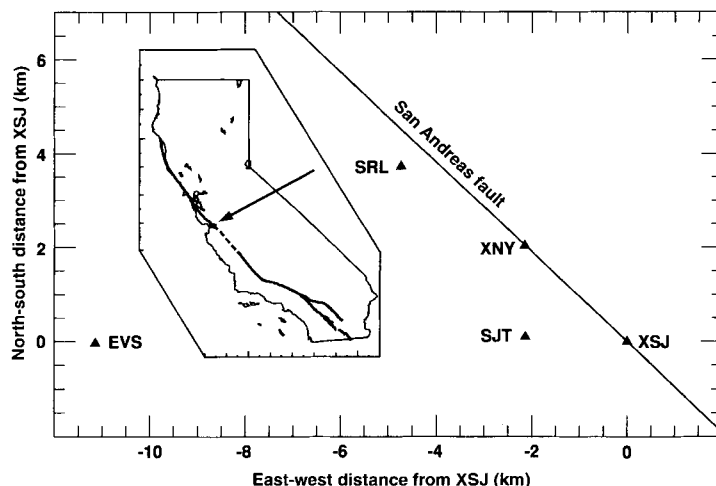
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EARTHQUAKES typically release stored strain energy on timescales of the order of seconds, limited by the velocity of sound in rock. Over the past 20 years, observations^{1–13} and laboratory experiments¹⁴ have indicated that rupture can also occur more slowly, with durations up to hours. Such events may be important in earthquake nucleation¹⁵ and in accounting for the excess of plate convergence over seismic slip in subduction zones. The detection of events with larger timescales requires near-field deformation measurements. In December 1992, two borehole strainmeters close to the San Andreas fault in California recorded a slow strain event of about a week in duration, and we show here that the strain changes were produced by a slow earthquake sequence (equivalent magnitude 4.8) with complexity similar to that of regular earthquakes. The largest earthquakes associated with these slow events were small (local magnitude 3.7) and contributed negligible strain release. The importance of slow earthquakes in the seismogenic process remains an open question, but these observations extend the observed timescale for slow events by two orders of magnitude.

Our study area (Fig. 1) is located at the transition between locked and stably sliding segments of the San Andreas fault in central California. The fault to the north ruptured in the great 1906 earthquake, and to the south, fault slip occurs through creep and small (local magnitude $m_L \lesssim 3$) earthquakes. Our borehole instruments are a Sacks–Evertson strainmeter¹⁶ (dilatometer) at site SRL (depth 138 m) and a Gladwin tensor (three-component) strainmeter¹⁷ at SGT (146 m). The first instrument measures volume change (dilatation). The second measures horizontal deformation in three directions at 120°. These measurements are combined to give areal strain and two orthogonal shear strains. The data are collected by the US Geological Survey (USGS) in Menlo Park via satellite digital telemetry¹⁸. SRL is sampled every 10 minutes and SGT every 18 minutes. Another dilatometer at EVS, more distant from the fault, is in highly fractured rock which

FIG. 1 Map of the location of borehole instruments SRL, SGT and EVS; of creepmeters XSJ and XNY, and of the San Andreas fault. The insert of California (large tick marks are for 1 degree of latitude and longitude) shows the site area (indicated by the arrow) and the main trace (heavy line) of the San Andreas fault. The dashed section south of the study area indicates the creeping part of the fault. Creepmeters span the fault and measure the relative displacement across it. Borehole strainmeters are located off the fault both to avoid installation in fault zone material and to allow sensitivity to deformation at depth on the fault.



gives low-quality data; these are not inconsistent with our models but are not valuable for constraining parameters. Surface slip on the fault near these instruments is monitored by two 10-m-long creepmeters that cross the fault obliquely^{19,20}.

In December 1992, the strainmeters recorded a strain excursion unique to the data since observations began in 1984. Figure 2 shows eight months of data from SRL and SGT starting about six months before the slow event. The large, slow strain change in December dominates the record. Also shown in Fig. 2 are the earthquakes located in the area approximately between SRL and SGT with magnitudes greater than or equal to 2.5.

Figure 3 shows 10 days of data (solid lines) from both instruments. These data have been linearly detrended, and tidal components and strain changes induced by atmospheric pressure have been removed²¹. Theoretical estimates of the ocean-loaded tidal amplitudes²² have been used for calibration. Both instruments have a constant response to strain over all periods of interest. The slow changes began with a relatively rapid change on 11 December followed by slow, exponential-like changes over about 8 days, interrupted by relatively rapid changes on 12 December and on 14 December. These changes over time are indicative of a series of slow events, which we have labelled A to E.

Creep data (Fig. 3) from sites XNY¹⁸ and XSJ¹⁹ provide qualitative constraints for modelling. Creep events are different from slow earthquakes; they are due to the nonlinear failure of the near-surface materials (of depths less than a few hundred metres), perhaps in response to deeper slip^{23–25}. At XSJ, there is no relative displacement across the fault until event E. At XNY, creep response to the deep slip was probably masked by left-lateral surface displacement, caused by heavy rain, which reversed to the tectonic right-lateral sense soon after event C. The slow strain event is not caused by precipitation; it is the only event of this type in 12 years of data (up to mid-1996), whereas there are comparable or larger amounts of rain every wet season in December–January.

One earthquake (m_L 3.1; number 1 in Figs 3, 4) occurred about two hours before the slow sequence began. Two earthquakes (m_L 3.3, 3.2; numbers 2, 3) occurred within the first and third sample intervals (on both instruments) showing slow strain changes. The next day two m_L 3.7 earthquakes (numbers 5, 6) took place during the first sample interval of large slow strain change on both instruments. Smaller earthquakes (including numbers 4 and 7) preceded and followed these more rapid changes. Coseismic strain changes for these earthquakes are negligible (a few nano-

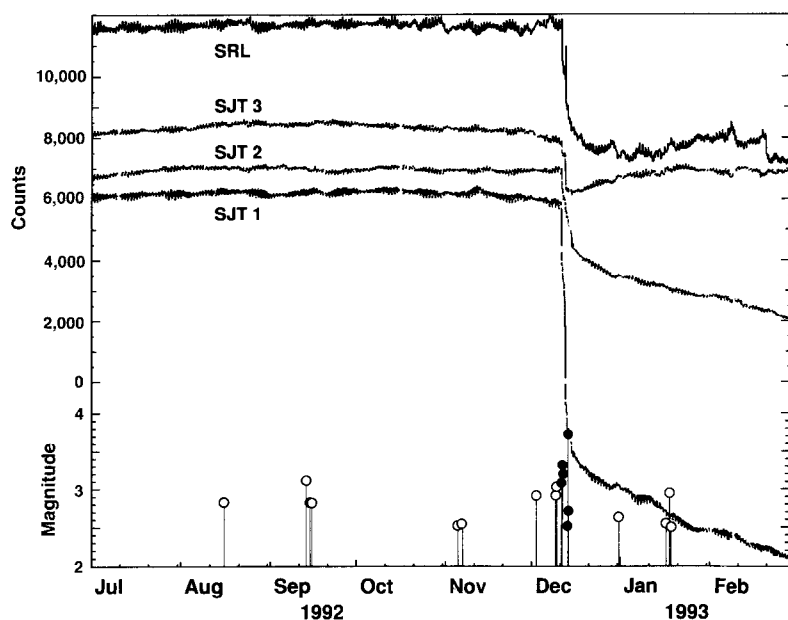


FIG. 2 Eight months of detrended data from SRL and from the three sensors of SGT. The sign of the data from SJT1 has been reversed. Short-term variations are the solid earth tides and some of the longer-term changes are due to variations in atmospheric pressure. The slow earthquake sequence in early December dominates the record. The range of the number of counts shows that the event is very well resolved. The lower part of the figure shows local earthquakes with magnitudes of at least 2.5; solid circles denote those that occurred during the time interval shown in Fig. 3. For about 10 days (see Fig. 3) the two strainmeters show excellent coherence. Slow deformation continued for about six months but the strain changes are less well correlated, suggesting that slow motion continued quasi-independently on smaller areas of the fault, or propagated past the along-fault limits given here (Fig. 4).

strain) compared with the observed strain changes (100–500 nanostrain) during those intervals. We include calculated²⁶ coseismic changes, but omitting them does not bias the model parameters for the slow events. Because of our sampling intervals (10 minutes for SRL, 18 minutes for SJT), we are unable to determine whether the earthquakes came before or after the initiation of the slow events. However, the absence of comparable seismicity, before and after this episode, is indicative of a causal connection between the occurrence of the slow events and earthquakes.

We restrict source models to pure right-lateral slip on areas of the San Andreas fault, taken as a vertical plane with strike N133.7E. We search for the simplest solutions that provide satisfactory agreement with the strain data and consistency with the creep observations. As the strain changes are slow, we use the expressions given by Okada²⁶ for the deformations produced by a dislocation in an elastic half-space. We incorporate these into an algorithm for computing quasistatic, strain-change time series (at instrument depths) attributable to a slowly propagating rupture and/or a slow slip rise time. From the relative amplitudes of the strain changes, it is clear that we must have slip sources that are concentrated on the fault between SJT and SRL.

Consider first the strain events E and C. Event E is similar to a number of previous slow events which were modelled by Gladwin *et al.*¹³ and we find a source (patch W in Fig. 4) with geometry similar to their model, although we have restricted the depth extent to 0.3 km. Slip of 5 mm, with slow rupture (average rupture velocity 0.2 ms^{-1}) to the southeast on W, provides good agreement with the strain changes at SJT (and with no change at SRL) and with the subsequent creep at XSJ. Our model calculations are shown in Fig. 3 as dashed curves.

Event C has a strain signal character at SRL which is different from that for the strains at SJT; reversal of strain direction requires rupture propagation which is slow compared with our sample interval of 10 minutes, such that a nodal line in the strain field passes through the station location as the source geometry changes. We have examined all possible model areas from just north of SRL to just south of SJT. The only viable solution found is slip of 2.8 cm on the patch marked Z; the rupture propagates slowly upwards (with exponentially decreasing rupture velocity, with average 0.35 ms^{-1}), resulting in a sign reversal of strain change at SRL and unidirectional changes at SJT.

For the remaining events, A, B and D, we minimize the number of free parameters by requiring a fixed source geometry with different slips for the different events. Amplitude ratios among the various strains cannot be satisfied by the simplest model of uniform slip over a single source area; we achieve good agreement by using adjoining areas X and Y (Fig. 4). We limit the top of Y to be 0.3 km from the surface, for consistency with lack of creep at XSJ until E; the top of X is 0.1 km deep. We have reasonable control of the extent along strike; the positions of the remote ends could be varied by about 1 km without significantly degrading the fit to the data. The boundary between X and Y could also be varied somewhat, but it probably could not be closer to XNY for the model to be consistent with the creep data. The top boundaries are well constrained; increasing their depth decreases the quality of the fit, and shallower depths conflict with the creep data. Our bottom depth control is much poorer. Both SJT and SRL are close to the fault and have little sensitivity to slip below about 4 km depth. We choose the minimum-area solution shown in Fig. 4 but equally good fits to the data could be obtained for sources with bottom extent anywhere in the depth range 4–8 km, or even deeper. Also sources of depths less than 4 km require less slip than those extending to 8 km because deeper parts of the source produce strains (at our sites) opposite in sign to those from the shallower parts. The quality of the fit decreases for depths less than 4 km. Our model (dashed curves in Fig. 3) has slip on X and Y of 3.7 and 2.9 mm, 5.6 and 3.4 mm, and 9.2 and 8.5 mm for events A, B and D respectively. The area of greatest slip is just above the cluster of larger earthquakes. Slow rupture propagation results in differences in shape between the recorded strains (for

example as for event C). Because all strain signals have similar waveforms for A, for B and D, the simplest model has a rupture propagation time which is short compared with our sample interval of 10 min; the time variation results from slip rise time. The exponential time constants are 40 min for A, 15 h for B and 43 h for D.

The sequence of events in the model is that there was an initial episode (A) of slow slip over all of X and Y, followed by slower slip (B) over the same area. Then slow rupture propagated upwards over Z (event C); there may have been continuing slip over all of X and Y during this interval although our model does not include it. Slip then continued (more slowly) over X and Y (event D) and during that time, failure on patch W produced event E. This sequence is the simplest model we can find to provide reasonable agreement with the data.

The data provide unambiguous evidence for a slow earthquake

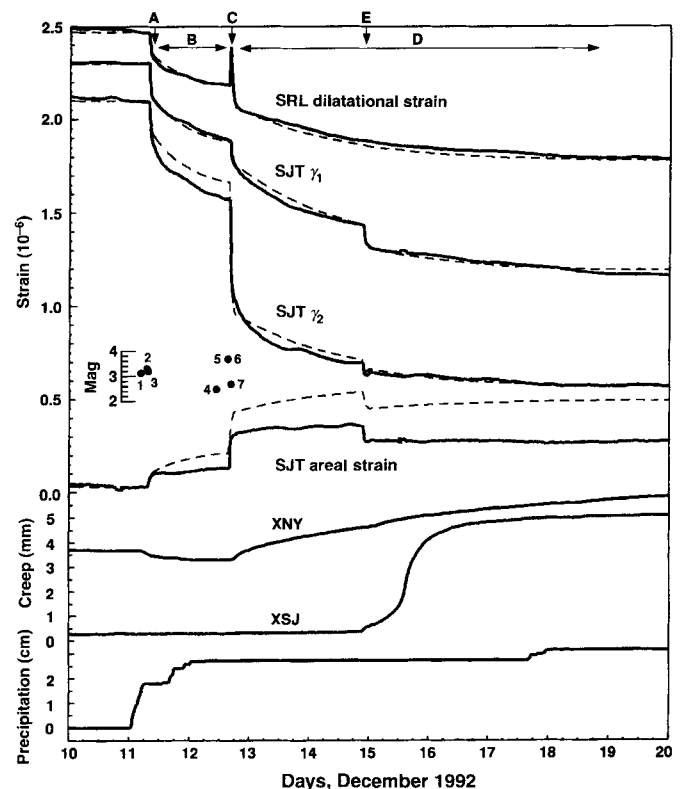


FIG. 3 Strain data (solid lines) from SRL and SJT for 10 days in December 1992 covering the duration of coherent slow changes. SRL records dilatational strain. From SJT we get γ_1 shear at N45 W (approximately fault-parallel) fault-normal shear γ_2 , and areal strain. Earth tides and strain changes induced by atmospheric pressure have been removed; some residual tidal variations remain, including small changes before the initiation of the slow sequence. Creep data (XNY and XSJ) and precipitation are also shown. Earthquake times and magnitudes are shown in the insert numbered in order of origin time; there are two m_L 3.7 events on 12 December. The labels A–E indicate slow events within the sequence. Dashed curves are results from model calculations. Calculated coseismic changes are included but are not significant. The results are not significantly affected by reasonable errors in the site locations or by the non-verticality of the fault. The fit to SRL dilatation and to γ_1 shear is essentially perfect. We could improve the fit to areal strain by changing the amount of slip on X and Y (see text and Fig. 4) for events A, B and D without decreasing the fit to dilatation and γ_1 , but the quality of the fit for γ_2 would decrease. This apparent discrepancy may be a result of using a homogenous Earth model in processing the data from the three sensors of SJT to produce the areal and shear strains. Preliminary finite-element modelling of the differences in material elastic constants on opposite sides of the fault indicates that area strain has been underestimated and that γ_2 has been overestimated.

sequence with significant complexity, not unlike that seen in regular earthquakes; such complex behaviour has not previously been observed for slow earthquakes. This event, with slip occurring over a period of about one week, extends the observed timescale for slow earthquakes by two orders of magnitude. We require slow slip over a large surface, with total moment equivalent to about a magnitude 4.8 earthquake. The occurrence of a slow earthquake on this part of the San Andreas fault may have some bearing on the nature of the transition from locked to creeping characteristic behaviour, but we cannot say exactly what this might be. The moment release of this event is small in comparison with the slip deficit proposed^{27,28} for the San Andreas fault to the north of our study area. Earthquakes as large as magnitude 4.6 have occurred in this area; the capability of the fault to generate both slow and regular earthquakes is similar to that reported by Sacks *et al.*⁶ for a fault in the Izu peninsula, Japan. Whereas the slowness of the Izu event could be ascribed primarily to slow rupture propagation, here most of the strain changes are attributed to slow slip rise time. Thus, not only can faults sustain ruptures over a wide range of timescales also the slowness can be in either or both of the rupture velocity and the time for slip to reach its final value. This slow sequence (with slip of a few centimetres) was accompanied by only small earthquakes, whereas the Izu slow event (slip of ~1 m) was followed by earthquakes as large as magnitude (m_b) 5.8. The slow precursor to the great (moment magnitude 9.5) 1960 Chile earthquake¹⁻⁴ was very large in extent, with slip of at least several metres.

These few observations seem to suggest a relation between the amount of slow redistribution of stress and the size of associated earthquakes, but additional observations of associated slow and regular earthquakes will be necessary before firm conclusions can be drawn. Unfortunately, slow events are difficult to detect; the total surface displacement generated by this slow sequence would have been barely detectable by Global Positioning System (GPS) receivers at our sites. As there are relatively few arrays of borehole strainmeters, and signal amplitudes decrease rapidly with increasing distance from the source, progress in determining the role of slow earthquakes in the seismogenic process is likely to be slow. □

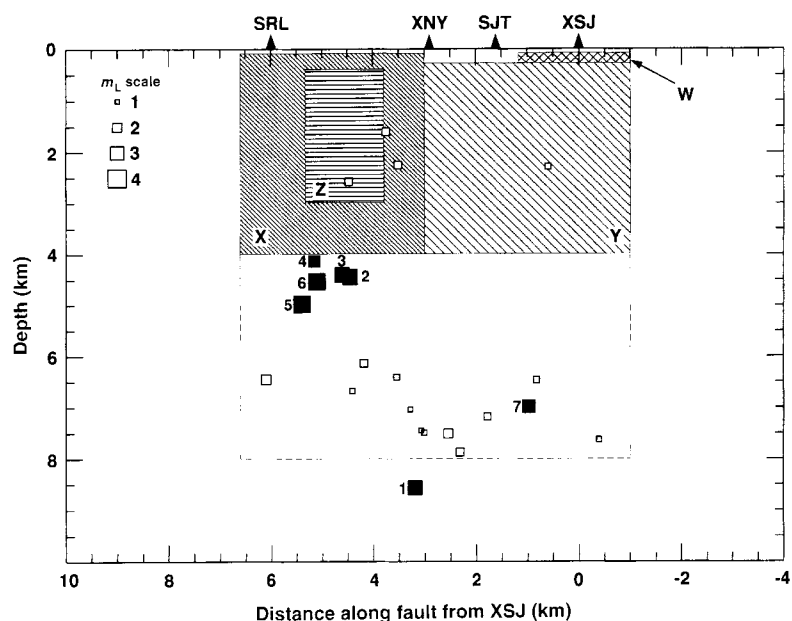


FIG. 4 Vertical section in the local strike direction of the San Andreas fault. Earthquakes are shown as filled squares for events with magnitudes ≥ 2.5 (numbered as in Fig. 3), and open squares for smaller events; areas of these events are much smaller than for the slow slip. XSJ and XNY show the locations of creepmeters; SRL and SJT sites are shown projected onto the fault. Shaded areas are those used for the model calculations. Aseismic slip in X and Y corresponds to strain events A, B and D. Aseismic slip on Z and W corresponds to events C and D respectively. Dashed lines indicate that the source depths for X and Y could extend to 8 km.

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ACKNOWLEDGEMENTS. We thank D. Myren for helping to operate the SRL station, S. Silverman and K. Breckenridge for maintaining data acquisition files, and G. Beroza for reviewing the manuscript.

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A Palaeocene proboscidean from Morocco

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UNTIL recently, the oldest known Arabo-African fossils of the elephant order (Proboscidea) were scarce *Moeritherium*-like remains from the Middle Eocene epoch of Mali¹ and Senegal². In 1984 the discovery in Algeria of *Numidotherium koholense*³ pushed back the record to the late Early Eocene. Here we report the discovery of a new genus in the late Palaeocene (Thanetian) epoch of Morocco (Ouled Abdoun Basin), about 7 million years older than *Numidotherium*. The new specimen is not only the oldest and smallest known proboscidean, but also the first modern ungulate from pre-Eocene strata. Though indirect data support an early eutherian radiation, close to the Cretaceous/

Received 28 March; accepted 17 July 1996.

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Tertiary boundary, this is also among the very few known occurrences of modern placental orders before the Eocene. Unexpectedly, it belongs to what is, according to current phylogenetic studies^{4,5}, one of the most derived eutherian orders, providing new evidence for a very early radiation of modern orders of placentals.

The species is documented by two maxillary fragments which are the first mammalian remains reported from the North African phosphates. Though unexpectedly small (about 10–15 kg), it is closely related to African proboscideans, especially to lophodont taxa such as *Numidotherium*³.

Family Numidotheriidae Shoshani & Tassy, 1992⁶
Phosphatherium escuilliei gen. et. sp. nov.

Holotype. PM2, left maxilla with $P^{3,4}$, $M^{1,2}$ (Fig. 1b).

Hypodigm. Holotype and PM1, fragment of left maxilla with dP^4 , M^1 (Fig. 1a, c); collections of Laboratoire de Paléontologie des Vertébrés, Université Paris 6, France.

Locality and Age. The material was obtained by F. Escuillie together with selachian teeth, from a fossil dealer. It came originally from phosphates of Ouled Abdoun Basin, Morocco. The discoverer and exact geographical origin are unknown; however, an analysis of the matrix of the specimens has confirmed its phosphatic nature and it has yielded selachian microteeth (Scyliorhinidae, Rhinobatidae, Dasyatidae). Selachian faunas and their accurate stratigraphic ranges have been extensively investigated in Ouled Abdoun and other Moroccan phosphate basins^{7–11}. Each biostratigraphic stage, and each level within the stages, are perfectly characterized by their species associations. The recovered selachian species association, which includes new marker taxa¹¹, indicates an unambiguous Thanetian age. It is characterized by the absence of Ypresian taxa and by the occurrence of the following Thanetian marker taxa^{10,11}: Scyliorhinidae: *Scyliorhinus* sp. A, Genus A sp. 1 and Genus B sp. 1 (ref. 11); Rhinobatidae: *Rhinobatos* sp. 11; Dasyatidae: *Dasyatis* sp. 1 (ref. 11). New information about the locality indicates that the fossils come from the Ouled Abdoun 'couche II' (quarrier terminology) which is indeed classically dated as Thanetian by the selachian species present⁷.

Diagnosis. Dental pattern close to *Numidotherium koholense*, especially in the true lophodont¹² upper molars which lack conules. Differs in the much smaller size (35–40% of *N. koholense* molar size), the more developed ectoloph, the centrocrista linked to a well (dP^4) or poorly (molars) developed mesostyle, the slightly less pronounced lophodonty of dP^4 and molars with a lower and mesodistally less compressed crown, the larger parastyle, the large postentoconule of dP^4 , the more developed metacone of the premolars, the shorter upper diastema and the absence of a deep submaxillary fossa (anterior part).

Only the upper dentition (dP^4 , $P^{3,4}$, $M^{1,2}$) and the maxilla were found (Fig. 1). They will be described elsewhere. On the basis of allometric scaling^{13,14} from $M^{1,2}$ size (holotype: M^1 , 8.65×8.80 mm; M^2 , 11.15×10.70 mm), the estimated body mass of the species was 10–15 kg.

The bilophodont molars, the distinct but weak centrocrista, the occurrence of a postentoconule and especially the anterior position of the orbit^{15,16}, as indicated by the position of the infraorbital foramen under the distinct suborbital process, are features of tethytheres (Proboscidea and Sirenia). Within tethytheres, the Moroccan species shows derived features that are found only among Arabo-African proboscideans: the true lophodont molars which lack any trace of conules, the developed distocrista, and the loss of P^1 (refs. 12, 15, 17–19). They exclude a special sirenian relationship within Tethytheria.

Several distinctive characters justify the designation of the new genus and species *Phosphatherium escuilliei*. *P. escuilliei* has an original collection of features that are probably primitive with respect to other proboscideans. The distinct centrocrista linked to a mesostyle in dP^4 and $M^{1,2}$ is a remnant of the dilambdodonty which is a symplesiomorphic feature of bilophodont ungulates,

namely of pantomesaxonians (perissodactyls, hyracoids, tethytheres and extinct relatives); it is known, for example, in perissodactyls, embrithopods and hyracoids. The dilambdodonty evolved early, in phenacodontid condylarths which share other significant features with pantomesaxonians²⁰. Such a vestigial dilambdodonty is seen in *Moeritherium* sp. from In Tafidet¹. The inflated postentoconule (dP^4), also known in *Moeritherium* and sirenians, may also be primitive (or secondary in proboscideans with three or more lophs). In *P. escuilliei*, the centrocrista, mesostyle and postentoconule are significantly more reduced in the molars than in dP^4 , in accordance with their probable primitive polarity. The reduction of these traits in the molars, together with the mesodistally more compressed crown and the more transverse

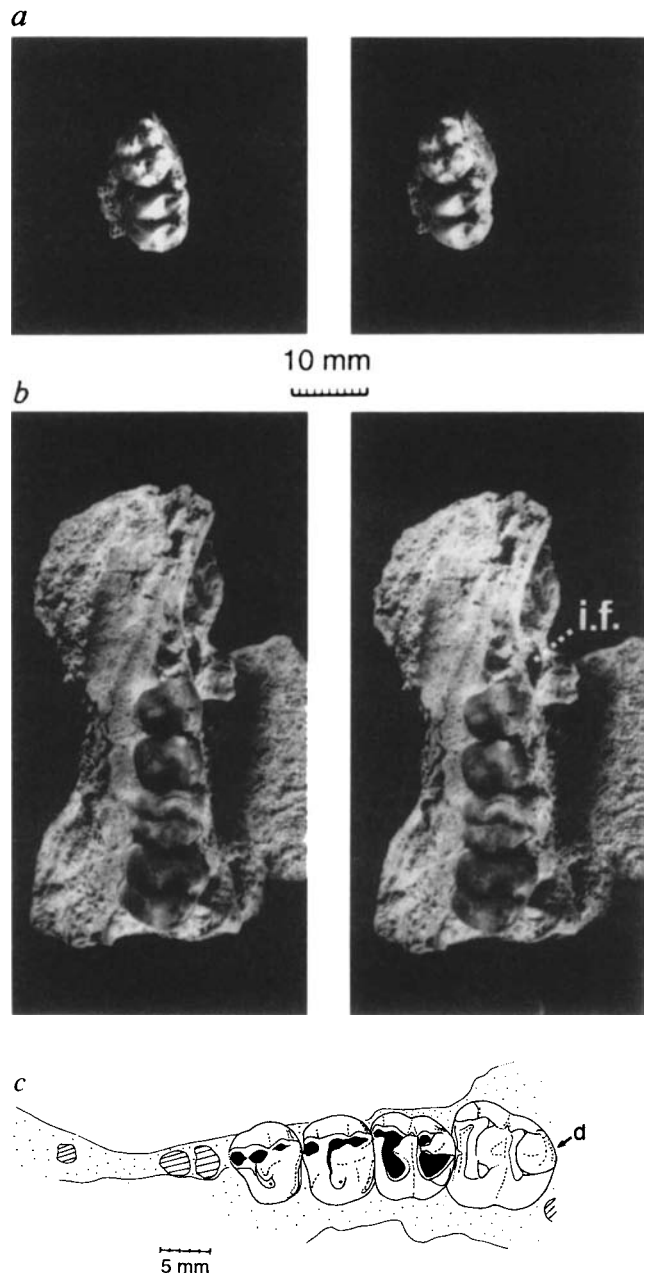


FIG. 1 *Phosphatherium escuilliei* gen. et. sp. nov. a, PM1, fragment of left maxilla with dP^4 and M^1 in occlusal view (stereophotographs). Scale bar, 10 mm. b, Holotype, PM2, left maxilla with $P^{3,4}$, $M^{1,2}$ and alveoli for C^1 and P^3 in occlusal view (stereophotographs). i.f., infraorbital foramen; scale bar, 10 mm. c, Sketch of the occlusal outline of the upper dentition preserved in the holotype PM2: $P^{3,4}$, $M^{1,2}$ and alveoli for canine, P^2 and M^3 (lingual root partially preserved). d, distocrista; scale bar, 5 mm.

lophs, is indeed related to the more pronounced lophodonty.

Within proboscideans, *P. escuilliei* is especially reminiscent of true lophodont taxa such as *Numidotherium*, *Barytherium* and deinotheres. Deinotheres are especially distinguished by their large size and their trilophodont M¹ and dP⁴. *Phosphatherium* more closely resembles *Barytherium*. It differs, however, from the latter in features that are common to *N. koholense*: presence of a small C¹; P^{3,4} submolariform with a metacone; molars less compressed, with trace of postentocone and without a lingual cingulum (may be present in *Barytherium*; P. Tassy, personal communication); molar lingual root not divided; and zygomatic process of the maxilla more posterior. In dental morphology, *P. escuilliei* is almost identical to *N. koholense*, despite a considerable size difference, and it is consequently included in the same family Numidotheriidae. However, as the only possible shared derived feature of *P. escuilliei* and *N. koholense* is the absence of a molar lingual cingulum³, the Numidotheriidae including *Phosphatherium* may be paraphyletic. An ancestor–descendant relationship between *P. escuilliei* and *N. koholense* is possible (Fig. 2); most of their differences (see diagnosis) result from the primitive nature of the former. *P. escuilliei* is the most primitive known lophodont proboscidean.

Moeritherium, though sharing notable features such as a large postentocone, is well distinguished by its bunolophodonty, that is, cusps transversely inflated and subdivided (para- and meta-

cone), a derived condition. Anthracobunids²¹ from the Indian subcontinent lack a postentocone and are more distant from *Phosphatherium* than moeritheres in premolar morphology and in dental formula. Their bunodont–bilophodont molars are more primitive than those of moeritheres. The relationships between anthracobunids are still unclarified. They may be primitive tethytheres^{15,16}, but are excluded here from Proboscidea.

Phosphatherium shows that the true lophodonty of proboscideans developed very early, and at a stage retaining a vestigial dilambdodonty. Such an early age of the lophodonty in proboscideans implies, according to their current phylogenies^{12,15,17–19}, that the *Moeritherium* lineage is very old. A new hypothesis²², which expresses a derived position for *Moeritherium* fits better with the age of fossil taxa. In this analysis of Proboscidea, as in the traditional one, bunolophodonty is primitive and lophodonty is derived. The age and morphology of *Phosphatherium* and other early primitive proboscideans such as *Numidotherium*, *Barytherium* and even deinotheres, in our view favour a possible true lophodont ancestral morphotype of Proboscidea, whatever the position of *Moeritherium* (Fig. 2a, b).

The discovery of *Phosphatherium* confirms the ancient African origin of Proboscidea. The role of Africa in the initial radiation of placentals may have been significant. The continent is thought^{23,24} to have been the source of main modern orders of large placentals such as hyracoids, perissodactyls and artiodactyls, and also of the primates and the extinct hyaenodontids. The antiquity of the eutherian population of Africa was previously suggested by the Adrar Mgorn locality^{25–29}, the only other Palaeocene record of mammals on the continent. The site has confirmed the African origin of primates³⁰, but not that of the large placentals which are absent there because of a taphonomic bias. The discovery of *Phosphatherium* indicates that the Moroccan phosphates might provide new significant information about the early history of modern placentals. According to current phylogenetic studies^{4,5}, the Proboscidea exemplify one of the last nodes in the branching sequence of placental orders. *Phosphatherium* provides new evidence for the very early and rapid radiation of modern orders of placentals, before the late Palaeocene epoch. □

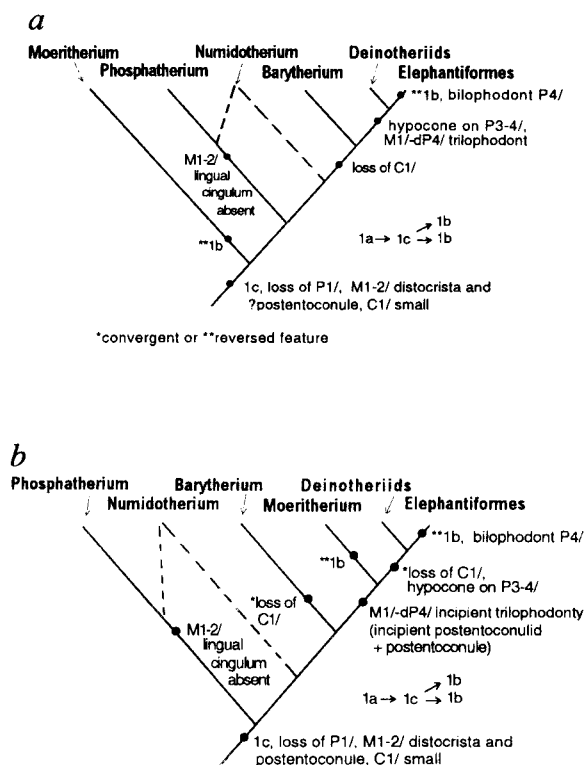


FIG. 2 Position of *Phosphatherium* in the traditional cladogram (a) of proboscideans^{14,17,19–21} and in the cladogram (b) which is an adaptation of a new analysis²⁴ of the derived position of *Moeritherium*. The new hypothesis is modified here by including *Barytherium* in the cladogram. Distribution of presumed derived features of the upper dentition as discussed in the text; non-proboscidean tethytheres such as the sirenians are among the more pertinent outgroups. We favour a true lophodont ancestral morphotype of Proboscidea, especially the hypothesis b. The latter implies striking reversals (for example conules), but it is supported by the morphology of earliest known proboscideans such as *Phosphatherium*, *Numidotherium* and *Barytherium*. These preliminary suggestions of the position of *Phosphatherium*, especially hypothesis b, require further testing by general parsimony analysis. 1a, primitive bunodont–bilophodont anthracobunid-like pattern (outgroup condition); 1b, bunolophodonty; 1c, true lophodonty.

Received 27 December 1995; accepted 8 July 1996.

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ACKNOWLEDGEMENTS. We thank P. Tassy for helpful discussions and unpublished information and N. Court for useful comments on the manuscript; E. Buffetaut improved the English version; photographs are by C. Abrial (CNRS).

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Minimal cost per twitch in rattlesnake tail muscle

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SOUND production is one of the most energetically costly activities in animals¹. Minimizing contraction costs is one means of achieving the high activation rates necessary for sound production (20–550 Hz) (refs 1–3) without exceeding energy supplies. Rattlesnakes produce a sustained, high-frequency warning sound by extremely rapid contraction of their tailshaker muscles (20–90 Hz) (refs 4, 5). The ATP cost per twitch is only 0.015 μmol ATP per g muscle per twitch during rattling, as measured by *in vivo* magnetic resonance. The reduced volume density of myofibre (32%) in tailshaker muscle is consistent with contraction cost being minimized (crossbridge cycling), in contrast to the contractile costs of vertebrate locomotory and asynchronous insect flight muscle. Thus tailshaker muscle is an example of sound-producing muscle designed for 'high frequency, minimal cost'. The high rates of rattling are achieved by minimizing contractile use of ATP, which reduces the cost per twitch to among the lowest found for striated muscle.

Tail shaking by the western diamondback rattlesnake (*Crotalus atrox*) produces a noise at frequencies audible to large mammals^{4,5}. Rattling is accomplished by unfused, high-frequency muscular contractions, made possible by an extraordinarily high density of sarcoplasmic reticulum and T tubules (26% of muscle volume) for rapid calcium cycling, and mitochondria (30% of muscle volume)⁴ for energy production. Sound production by the tailshaker group of muscles is 'all-or-none': each muscle functions as a single motor unit. Consequently, oxygen uptake parallels rattling frequency at all temperatures, resulting in a constant quantity of oxygen consumed per twitch (and hence per rattle), independent of frequency and temperature⁴.

We determined the contractile energetics of the tailshaker muscle under normal and electrically activated rattling, by using magnetic resonance spectroscopy on ten snakes. Normal and activated rattling causes reciprocal changes in phosphocreatine and inorganic phosphate (P_i) without a detectable change in ATP, as shown by the phosphorus spectra of the tailshaker muscle during rest and rattling (Fig. 1a). During rattling, the phosphocreatine breakdown is initially linear, followed by a decreasing slope that reflects activation of aerobic and glycolytic ATP synthesis (Fig. 1b). Confirmation that ATP synthesis is small during the initial phosphocreatine breakdown period^{7,8} comes from the increase in cellular pH (0.25 ± 0.1 (s.e.); one-tailed, paired *t*-test, $P > 0.025$, $n = 8$) and the low oxidative ATP synthesis (0.0017 ± 0.0003 (s.e.) μmol ATP per g muscle per twitch; $n = 9$) estimated from the ADP and P_i levels for the first spectrum in rattling (see legend to Fig. 1). The difference in phosphocreatine level between the last resting and first rattling spectrum (4.5 s in Fig. 1b) yielded the ATP use rate, which increased threefold with natural rattling frequency (25 Hz at 10 °C to 70 Hz at 30 °C; Fig. 2a). The ATP use rate per twitch (0.015 ± 0.001 (s.e.) μmol ATP per g muscle per twitch; $n = 19$) was constant for all conditions (Fig. 2b), and represents 50% of the whole-body energy consumption ($0.037 \mu\text{mol}$ ATP per g muscle per twitch for $\text{ATP}/\text{O}_2 = 6$)⁴.

This cost per twitch is among the lowest reported for any skeletal muscle, and is a small fraction of that reported for locomotory muscles from rodents of similar size ($0.26 \mu\text{mol}$ ATP per g muscle per twitch)⁹. Tail-shaking also has a lower cost per

twitch than that reported for many insects, the flight- and sound-producing muscles of which are also fully activated, completely aerobic and comprise nearly the entire sink for O_2 during activity¹⁰ (Fig. 3). The cost per twitch reaches a minimal value for flying insects at high wing-beat frequencies (>60 Hz; synchronous fliers, $1.25 \mu\text{mol}$ per g muscle per twitch¹¹; asynchronous fliers, $0.3 \mu\text{mol}$ ATP per g muscle per twitch), with rattling being among the lowest in cost per twitch ($0.015 \mu\text{mol}$ ATP per g muscle per twitch).

Asynchronous muscle has minimal activation (Ca^{2+} cycling) costs, so the lower cost per twitch in the tailshaker muscle indicates that rattling has minimized contractile costs relative to those of muscle activation. Support for minimized contractile performance at high frequencies in synchronous muscle comes from the low efficiency of converting chemical energy into mechanical work. In locust (*Schistocerca americana*) muscle, the low efficiency at 20 Hz is consistent with 70% of costs due to

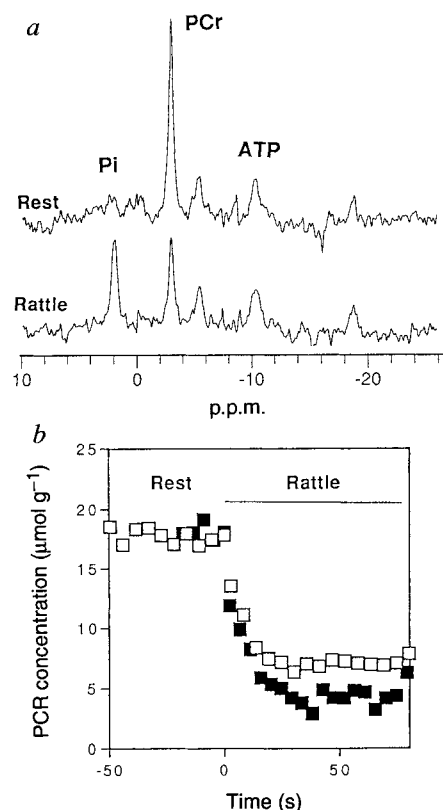


FIG. 1a, Experimental spectra in a single snake showing the changes from rest to rattling in phosphocreatine (PCr) and inorganic phosphate (P_i), but no detectable change in ATP. b, PCr concentration through the experiment under normal (open squares) and electrically stimulated (filled squares) rattling. The line indicates the period of rattling. The concentration with electrical stimulation is lower owing to reduced blood flow resulting from simultaneous activation of all tailshaker muscles. Snakes were elongated in a clear plastic tube in a horizontal bore GE Omega 4.7 Tesla spectrometer, with either a helmholz or solenoidal coil surrounding the tailshaker muscles. PCr and ATP peak areas were determined from fully relaxed spectra on resting tailshaker muscle⁸, and PCr/ATP did not vary with temperature (4.8 ± 0.92 (s.d.), $n = 10$). High-performance liquid chromatography measurements of tailshaker muscle yielded an ATP concentration of $3.8 \mu\text{mol}$ per g wet mass (± 0.75 s.e., $n = 3$) and total creatine level of $24.4 \mu\text{mol}$ per g wet mass (± 0.15 s.e.)²⁴, from which were calculated PCr using $\text{PCr}/\text{ATP} = 4.8$ and ADP from the creatine kinase reaction²⁵. Muscle activation was achieved by means of needle electrodes placed under the skin on either side of the motor neurons innervating the tailshaker muscles. We found the maximum-amplitude electromyograph at 25 V and 0.5 ms duration, but used 50 V to ensure supramaximal activation of rattling.

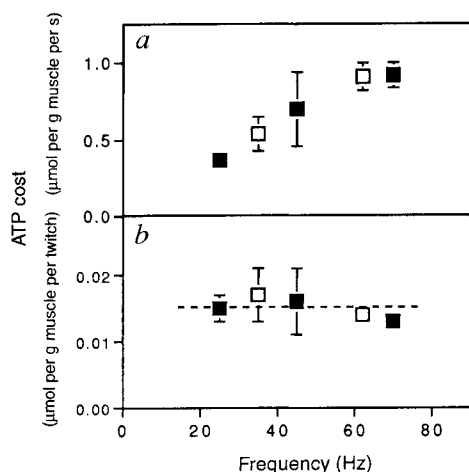


FIG. 2 ATP utilization of normal (open squares) and electrically stimulated (filled squares) rattling for the tailshaker muscle as a function of frequency. *a*, ATP use rate; *b*, ATP per twitch. The ATP use rates were determined from the change in PCr concentration between the last resting spectrum and the first spectrum during rattling by gating the magnetic resonance acquisitions to the onset of rattling or electrical stimulations⁹. We estimated the oxidative ATP synthesis rate from the measured Pi and calculated ADP levels²⁶, and oxidative rate of isolated mitochondria for the volume in tailshaker muscle²⁷, as previously described²⁶. Each sample is the mean of 3–5 snakes; vertical bars, s.e.

activation¹⁵, compared with 20–30% for vertebrate muscle¹⁶. The nearly equal volumes of sarcoplasmic reticulum (26%) and myofibril (32%) in the tailshaker muscle⁴, compared with 5% sarcoplasmic reticulum and >50% myofibre in vertebrate and insect muscle^{12,17}, further support the higher activation and lower contractile costs of rattling.

A minimized cost of contraction during sound production is consistent with the reduced mechanical performance per twitch observed at high frequencies^{12,18}. Muscle strain (Δ length/length) during sound production is among the lowest recorded (2%)¹⁹, indicating that there are very few crossbridge cycles (1–4 crossbridge cycles per twitch)^{20,21}, and low ATP use per twitch. Muscle stress (force/area) is also minimized, as shown by the lower force production in katydid muscle during sound production than in flight¹⁹. A further reduction in the muscle stress and the number of cycling crossbridges is expected in tailshaker muscles and other muscles specialized for sound production, on account of the lower fraction of muscle volume composed of myofibres (20–30%) (refs

2, 4, 22, 23) compared with vertebrate locomotory muscle (>70%)¹⁷ or asynchronous insect flight muscle (>50%)¹². These stresses and strains are lower because of the minimal force production needed for the rapid twitches of sound-producing muscle, compared with the high, sustained force production characteristic of striated muscle. Thus muscles designed for sound production have in common minimized contractile costs, resulting from operating close to the contractile limit of muscle strain (1–4 crossbridge cycles per twitch), and a reduced muscle stress (number of cycling crossbridges per unit area), resulting from a low myofibrillar fraction of muscle. That animals as unrelated as rattlesnake and insects share these features is suggestive of a strong selective pressure modifying the design of striated muscle for sound production. The rattlesnake tailshaker muscle is an extreme example of muscle designed for 'high frequency, low force', with the result that the cost per twitch may be the lowest of any striated muscle. □

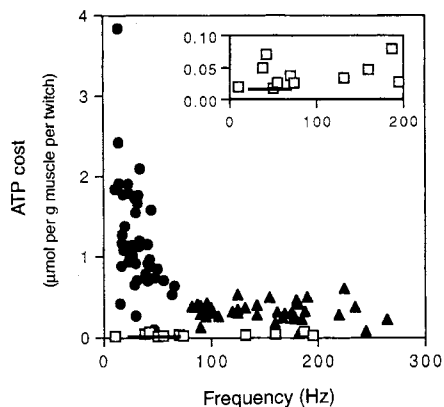


FIG. 3 ATP per twitch as a function of contraction frequency for the rattlesnake (bold line) and data from the literature: sound-producing insects (open squares; refs 1, 3); and flying insects with synchronous muscles (filled circles; ref. 11) and with asynchronous muscles (filled triangles; refs 1, 12–14). The inset shows sound-producing muscle only. Values are expressed per muscle mass for the tailshaker muscle, and assume that flight- and sound-producing muscles are 15% of body mass in insects¹³. Frequency is the rate of tailshaking (rattlesnake), calling (sound producers) or wingbeats. ATP use was determined from oxygen consumption using 22.4 ml (standard temperature and pressure, dry) per mmol, and $P/O_2 = 6$ (ref. 28).

Received 29 April; accepted 17 July 1996.

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ACKNOWLEDGEMENTS. We thank R. Gronka, R. Hedges, M. Kushmerick, S. Nichols, J. O'Reilly, R. O'Reilly, E. Shankland and R. Stuppard for contributions. This work was supported by grants from NSF, NIH and the Royalty Research Fund of the University of Washington.

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Role of tissue factor in embryonic blood vessel development

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Tissue factor, a member of the cytokine-receptor superfamily and high-affinity receptor and cofactor for plasma factor VII/VIIa (ref. 1), is the primary cellular initiator of blood coagulation. It is involved in thrombosis and inflammation associated with sepsis, atherosclerosis and cancer², and can participate in other cellular processes including intracellular signalling³, metastasis⁴, tumour-associated angiogenesis⁵, and embryogenesis⁶. Here we report that inactivation of the tissue factor gene (TF) results in abnormal circulation from yolk sac to embryo beyond embryonic day 8.5, leading to embryo wasting and death. Vitelline vessels from null mice were deficient in smooth-muscle α -actin-expressing mesenchymal cells, which participate in organization of the vessel wall. This implies that tissue factor has a role in blood-vessel development.

Targeted disruption of the *TF* gene was accomplished by deleting transcriptional and translational start signals (Fig. 1a). Genotyping of offspring (Fig. 1b) from *TF*^{+/-} mice indicated that the targeted *TF* allele was inherited in a mendelian pattern, but that *TF*^{-/-} embryos died *in utero* between embryonic days (E) 9.5 and 10.5 (Table 1). Of 350 offspring, only one *TF*^{-/-} pup survived to gestation. *TF* messenger RNA (Fig. 1c) and procoagulant activity (see Supplementary Information) were undetectable in E9.5 *TF*^{-/-} embryos and embryonic fibroblasts. At E8.5, *TF*^{-/-} conceptuses exhibited no morphological defects, developed intra-embryonic vessels and yolk sacs with numerous islands, filled with nucleated red blood cells, and developed vitelline vessels that connected to the embryo (see Supplementary Information). At E9.5, wasting of *TF*^{-/-} embryos was observed with variable onset and penetrance (Table 1). Despite *TF* expression in several normal *TF*^{+/-} embryonic tissues⁶, no specific morphological abnormalities were identifiable within E9.5 *TF*^{-/-} embryos. Because the vitello-embryonic circulation seemed abnormal, necrosis may have resulted from ischaemia secondary to the defective vitello-embryonic circulation and wasting, as had been observed previously in embryos deficient in transforming growth factor (TGF)- β (ref. 7). In the more severely affected *TF*^{-/-} embryos, necrosis of embryonic tissues was extensive.

Macroscopic analysis indicated that two-thirds of

the yolk sacs of E9.5 *TF*^{-/-} embryos lacked large vitelline vessels, and instead possessed an irregular plexus of enlarged capillaries (Fig. 2a, b). Although blood circulated within the less severely affected embryos, no blood flow was observed in the yolk sac. Collections of nucleated (embryonic) blood cells in the extracoelomic cavity were observed in the more severely affected embryos with resultant pallor and anaemia. Haematopoiesis in E8.5 *TF*^{-/-} embryos was normal, and the haematopoietic stem-cell proliferative capacity and the expression of the early haematopoietic markers GATA-1 (ref. 8) and β -haemoglobin-1 (ref. 8) RNA was similar in E9.5 *TF*^{+/-} and *TF*^{-/-} yolk sacs (see Supplementary Information). This suggests that the anaemia observed was consistent with extravasation of blood cells from the abnormal yolk-sac vessels. Approximately one-third of the *TF*^{-/-} embryos were normal at E9.5, but nearly all were abnormal at E10.5 (Table 1).

Microscopic analysis of abnormal *TF*^{-/-} yolk sacs indicated a lack of vitelline vessels and enlarged yolk-sac capillaries that appeared to fuse with each other in a disordered plexus (Fig. 2c-g). This was attributable to disintegration or defective formation of contacts between endodermal and mesothelial cell layers in the avascular zones, with resultant separation of both layers and fusion of adjacent vessels. Frequently, strands of acellular material formed the only residual connection between these cell layers (Fig. 2g), resulting in a seemingly confluent vascular bed with occasional 'blood lakes' (Fig. 2f). Vascular defects in the yolk sac preceded wasting and necrosis of the *TF*^{-/-} embryos (see Supplementary Information). Defects in the yolk-sac vasculature were also observed in embryos still embedded in their decidua. To exclude the possibility that defects in the vitelline vasculature and embryo wasting resulted from defects in communication between mother and fetus, *TF*^{+/-}, *TF*^{+/-} and *TF*^{-/-} embryos at E8.5 were cultured *in vitro* for 24 h. *TF*^{+/-} and *TF*^{+/-} embryos developed normally without signs of necrosis or vascular yolk-sac abnormalities (Fig. 2h), but *TF*^{-/-} yolk sacs were pale, anaemic, contained an irregular confluent vascular plexus ('blood lakes') and lacked vitelline vessels (Fig. 2i, j). Accumulation of blood in the extra-coelomic cavity and enlargement of the pericardial sac were observed only in the most severely affected embryos (Fig. 2j). Microscopic analysis of the yolk sac revealed an enlarged and disordered capillary plexus and the absence of properly developed vitelline vessels (Fig. 2k, l). As with *in vivo* studies, the vascular defects preceded embryo wasting (see Supplementary Information), and three of seven cultured *TF*^{-/-} yolk sacs and embryos appeared visually normal.

We used several methods to demonstrate that the vascular defects in *TF*^{-/-} yolk sacs were not due to obvious functional or morphological defects of endoderm or endothelial cell function.

TABLE 1 TF genotypes and corresponding phenotypes

Age and phenotype	Genotype		
	<i>TF</i> ^{+/-}	<i>TF</i> ^{+/-}	<i>TF</i> ^{-/-}
E8.5	11 (35%)	12 (39%)	8 (26%)
E9.5	39 (27%)	69 (48%)	36 (25%)
E normal, YS normal	9/9 (100%)	7/7 (100%)	6/7 (35%)
E normal, YS abnormal			2/17 (12%)
E necrotic, YS abnormal			4/17 (24%)
E necrotic, YS necrotic			5/17 (29%)
E10.5	6 (15%)	21 (51%)	14 (34%)*
E necrotic, YS necrotic			14/14 (100%)
>E14.5	30 (38%)	49 (61%)	1 (1.3%)*

Data represent the number of embryos (expressed as a percentage of total) from *TF*^{+/-} breeding pairs with their respective phenotype, as evaluated by macroscopic and microscopic analysis. Abnormality (wasting) and necrosis were graded in 17 embryos (E) and yolk sacs (YS). An example of a normal embryo with an abnormal yolk sac is represented in the Supplementary Information.

* A small number of *TF*^{-/-} embryos survived beyond E10.5 but died around E13.5 to E14.5 (cause unknown).

This was verified by reverse transcription-polymerase chain reaction (RT-PCR), by *in situ* hybridization and immunocytochemical analysis of specific markers of endoderm cells (E-cadherin⁹, HNF-4 (hepatocyte nuclear factor)¹⁰, α -fetoprotein, VEGF (vascular endothelial growth factor)¹¹ or endothelial cells (the receptor tyrosine kinases flk-1, tie-1, and tek-1 (ref. 11), VE-cadherin¹², PECAM¹² (platelet endothelial cell adhesion molecule)), by electron microscopy, by analysis of proliferation and apoptosis, by functional analysis of nutritional uptake¹³, and by *in situ* plasmin activity (a possible mediator of haemangioma cyst

formation) (see Supplementary Information). The structural defect was not attributable to necrosis or under-representation of visceral endoderm, endothelial or mesothelial cells, but was consistent with a paucity of interstitial mesenchymal cells (pericytes). Nuclear counts of mesenchymal cells per optical field at $\times 3,000$ magnification in three yolk sacs revealed 2.1 ± 0.2 nuclei (mean $\pm$ s.e.m.; $n = 40$ fields) in the $TF^{+/+}$ yolk sacs, and only 0.4 ± 0.07 nuclei ($n = 56$ fields) in the $TF^{-/-}$ yolk sacs ($P < 0.0001$). Smooth-muscle α -actin¹⁴ was expressed by fewer cells in the proximal, and was undetectable in the distal, vitelline

a Genomic map

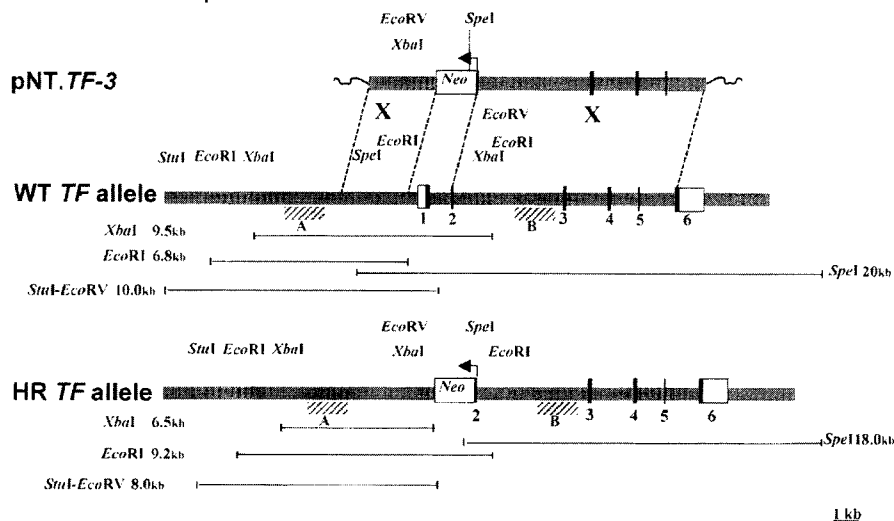


FIG. 1 Disruption of the *TF* gene and RNA analysis of the targeted allele. **a**, The targeting vector pNT.TF, the wild-type (WT) *TF* allele, and the homologously recombined (HR) *TF* allele with expected restriction fragments. Shaded bars, introns; open boxes, untranslated regions; filled boxes, coding regions; hatched boxes under the genomic structure, probes used for Southern blotting. Probe A is a 2.3-kb *HindIII*, and probe B is a 1.1-kb *AccI*-*XbaI* fragment. **b**, Southern blotting of *EcoRV*- and *StuI*-digested DNA from E9.5 $TF^{-/-}$, $TF^{+/+}$ and $TF^{+/-}$ embryos, hybridized with probe A, revealing 10-kb WT and 8.0-kb mutated fragments. **c**, Northern blotting of RNA from $TF^{+/+}$, $TF^{+/-}$ and $TF^{-/-}$ fibroblasts of E9.5 embryos, hybridized with a *EcoRV*-*SacI* murine *TF*-specific probe²³, revealing a 1.8-kb mRNA prominent in $TF^{+/+}$ cells, diminished in $TF^{+/-}$, and absent in $TF^{-/-}$ cells. The unstripped blot was rehybridized with an *hprt*-specific probe.

b Southern blot

c Northern blot

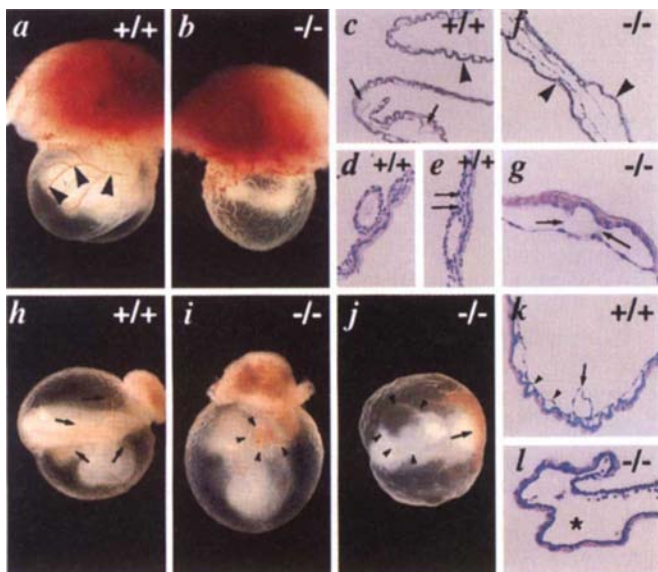
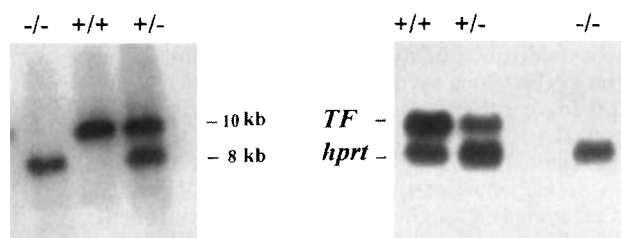
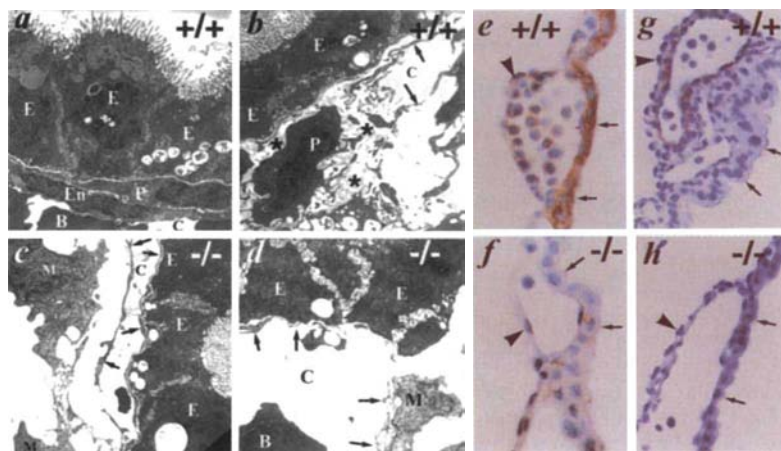


FIG. 2 **a, b**, E9.5 $TF^{+/+}$ (**a**) and $TF^{-/-}$ (**b**) yolk sacs revealing large, blood-filled vitello-embryonic vessels (arrowheads) in the $TF^{+/+}$, and an irregular vascular plexus without vitelline vessels in the $TF^{-/-}$ yolk sac. **c–g**, E9.5 $TF^{+/+}$ (**c–e**) and $TF^{-/-}$ (**f, g**) yolk sacs revealing multiple capillaries (**e**, and arrowheads in **c**) and vitelline vessels (**d**, and arrows in **c**) in $TF^{+/+}$ yolk sacs. Arrows in **e** indicate mesenchymal cells/pericytes. In $TF^{-/-}$ yolk sacs, yolk-sac vessels are abnormal (**f**) with strands of acellular material connecting endoderm and mesothelium (arrows in **g**). Endoderm, endothelial and mesothelial cells seem normal, but mesenchymal cell accumulation is reduced (**g**). **h–j**, *In vitro*-cultured E9.5 $TF^{+/+}$ (**h**) and $TF^{-/-}$ (**i, j**) embryos revealing vitelline vessels (arrows in **h**) and capillaries in $TF^{+/+}$ but 'blood lakes' (arrowheads in **i**) and a confluent disorganized vascular plexus (**j**) in $TF^{-/-}$. An enlarged pericardial sac and blood in the extracoelomic cavity (arrow in **j**) are visible. **k, l**, *In vitro*-cultured E9.5 $TF^{+/+}$ (**k**) and $TF^{-/-}$ (**l**) yolk sacs revealing multiple capillaries (arrowheads in **k**) and vitelline vessels (arrow in **k**) in $TF^{+/+}$, and an enlarged confluent vascular sac (asterisk) in $TF^{-/-}$ yolk sacs. The embryo is normal in both genotypes (see Supplementary Information).

FIG. 3 a–d, Electron micrographs of E9.5 $TF^{+/+}$ (a, b) and $TF^{-/-}$ (c, d) yolk sacs. In $TF^{+/+}$, visceral endoderm (E) is separated from endothelium (En) by interstitial mesenchymal cells (pericytes, P); $TF^{-/-}$ contains fewer mesenchymal cells/pericytes. In $TF^{+/+}$ (b), a capillary (C) with adjacent avascular connection between endoderm and mesothelial layers and abundant extracellular matrix (asterisks) surrounding an interstitial mesenchyme cell/pericyte is observed. $TF^{-/-}$ (d) is characterized by lack of interstitial mesenchymal cells and reduced matrix, but endoderm and endothelial cells are normal. M, mesothelial cell; C, capillary lumen; B, blood cells; arrows, endothelial cell processes. Magnification, $\times 7,000$. e–h, Immunostained E9.5 $TF^{+/+}$ (e, g) and $TF^{-/-}$ (f, h) yolk sacs revealing absent TF and reduced smooth-muscle α -actin in $TF^{-/-}$ vitelline vessels. Arrows, visceral endoderm cells; arrow-head, endothelial cells.



vessels of $TF^{-/-}$ yolk sacs (Fig. 3g, h, and Supplementary Information). The reduction of α -actin correlated with the extent of vascular defects. Because α -actin was undetectable at E8.5 but increased at and beyond E9.5 in these vessels (see Supplementary Information), TF deficiency seemed to compromise vascular development at a critical time when vessels began to develop a 'muscular' wall in response to physiological challenges such as blood pressure load. Decreased vimentin¹⁴ and syndecan expression in $TF^{-/-}$ yolk-sac capillaries and vitelline vessels paralleled the paucity of mesenchymal cells (see Supplementary Information). Electron microscopy identified fewer mesenchymal cells with less-abundant associated extracellular matrix in the $TF^{-/-}$ yolk sacs (Fig. 3a–d). The residual mesenchymal cells were predominantly associated with the endodermal layer, consistent with the greater reduction of syndecan expression at this site (see Supplementary Information). Fibrin was not observed in the $TF^{+/+}$ yolk sac by electron microscopy. Importantly, TF was expressed in the visceral endoderm cells of the yolk sac (Fig. 3e, f), consistent with TF having a role in yolk-sac vessel development. Thus we speculate that $TF^{-/-}$ yolk-sac vessels are fragile, and that their coalescence and fusion results in formation of an abnormal vascular bed.

In conclusion, $TF^{-/-}$ embryos undergo fatal wasting, which is attributable to defective yolk-sac vessel development and vitello-embryonic circulation. The proposed fragility of the yolk-sac vessels seems to be related not to dysfunction of the endoderm or endothelial cells, but to a defect in mesenchymal cell/pericyte accumulation and function, and to hypoplasia of the developing 'muscular' wall of the primitive vessels. Immature $TF^{-/-}$ yolk-sac vessels seem to respond inappropriately to the circulatory demands, which increase significantly at and beyond E9.5 and which are normally accommodated by pericyte and matrix accumulation and organization. Loss of perivascular pericytes has previously been related to the formation of micro-aneurysms in

diabetic retinopathy¹⁴ that are similar to the 'blood lakes' in $TF^{-/-}$ yolk sacs. That the extra-embryonic vasculature is more affected than the intra-embryonic vasculature could be related to poorer structural support by the surrounding tissues in the yolk sac than in the embryo, and to greater and earlier expression of TF and smooth-muscle α -actin in the vitello-embryonic and vitelline vessels than in the large embryonic vessels (see Supplementary Information), suggesting an earlier requirement of TF for development of the vitelline vessels. Whether TF exerts its role in vessel development by direct signalling, through the generation of downstream coagulation proteinases such as for factor Xa¹⁵ or thrombin¹⁶, or by undefined functions important for mesenchymal cell proliferation, localization and development¹⁷, remains to be determined. The embryonic lethality observed in $TF^{-/-}$ embryos resembles the phenotype of 50% of thrombin receptor-deficient embryos¹⁶, but contrasts to the bleeding phenotype of fibrinogen-deficient neonates¹⁸, suggesting a haemostasis-independent role for TF in vessel development. □

Methods

DNA and RNA analysis. All methods have been described previously^{19,20}. For construction of the targeting vector, a 2.5-kb 5'-flanking *HindIII* and an 8.0-kb 3'-flanking *EcoRV*–*SacI* fragment of the murine *TF* gene (from a 129 genomic lambda phage library) were cloned into pPNT in the opposite orientation to *neo*. For RNA analysis, confluent embryonic fibroblasts were serum starved for 48 h and then treated with 20% fetal bovine serum and cycloheximide ($10 \mu\text{g ml}^{-1}$) for 4 h. Embryos were genotyped by Southern blot or PCR analysis (for sequence of primers, see Supplementary Information) on yolk sac-derived DNA, or on DNA retrieved from microscopic slides, after deparaffination and proteinase K digestion.

Morphological analysis. $TF^{+/+}$ and $TF^{-/-}$ embryos and yolk sacs were dissected at E9.5, fixed in 4% formalin, paraffin-embedded and stained with haematoxylin²⁰. Embryo cultures were performed as described²¹. Immunohistochemical analysis^{19,20} and electron microscopy²² were performed as described previously.

Received 11 April; accepted 19 July 1996.

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ACKNOWLEDGEMENTS. This work was presented at the XVth Congress of the International Society of Thrombosis and Haemostasis, Jerusalem, 11–16 June 1995. We thank H. Kataoke and S. Nishikawa for the α -1 antibody; O. Huber for the E-cadherin antibody; A. Simeone, V. Attenburrow, A. Bouché, I. Cornelissen, M. De Mol, B. Hermans, A. Janssens, L. Kieckens, V. Van Gerven, A. Vanden Hoeck, C. Tränker, I. Peterson and U. Albrecht for technical help; and E. Adamson, C. Banka and M. Hanks for discussions. This work was supported in part by an NIH grant P01 HL 16411 (N.M. and T.E.), and performed during the tenure of an established investigatorship from the American Heart Association (N.M.).

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A neuronal learning rule for sub-millisecond temporal coding

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A PARADOX that exists in auditory and electrosensory neural systems^{1,2} is that they encode behaviourally relevant signals in the range of a few microseconds with neurons that are at least one order of magnitude slower. The importance of temporal coding in neural information processing is not clear yet^{3–8}. A central question is whether neuronal firing can be more precise than the time constants of the neuronal processes involved⁹. Here we address this problem using the auditory system of the barn owl as an example. We present a modelling study based on computer simulations of a neuron in the laminar nucleus. Three observations explain the paradox. First, spiking of an 'integrate-and-fire' neuron driven by excitatory postsynaptic potentials with a width at half-maximum height of 250 μ s, has an accuracy of 25 μ s if the presynaptic signals arrive coherently. Second, the necessary degree of coherence in the signal arrival times can be attained during ontogenetic development by virtue of an unsupervised hebbian learning rule. Learning selects connections with matching delays from a broad distribution of axons with random delays. Third, the learning rule also selects the correct delays from two independent groups of inputs, for example, from the left and right ear.

Barn owls use interaural time differences for azimuthal sound localization^{10,11}. They can locate sounds, and hence prey, with a precision of 1–2 degrees¹², a capability that requires a time resolution of less than 5 μ s (ref. 11). The key process in the auditory system is 'phase locking': spikes occur preferentially at a certain phase, termed the 'mean phase', of a stimulating tone, at frequencies up to 8 kHz (ref. 13). To understand the high degree of temporal precision in phase locking, we must consider both the typical duration (τ_s) of the synaptic input current and the membrane time constant (τ_m). For auditory neurons in the chicken^{14,15}, time constants of synaptic input are in the range of 200 μ s. Even though the passive membrane time constant is about 2 ms (ref. 14), the effective membrane time constant (see Fig. 1, methods) of magnocellular and laminar neurons is shorter than 200 μ s because there is an outward rectifying current that is activated above the resting potential¹⁴. Such outward rectifying currents are commonly found in phase-locking neurons of the auditory system^{16,17}. As a result of the short time constants, the width of single excitatory postsynaptic potentials (e.p.s.ps) in chicken is about 500–800 μ s at half maximum amplitude¹⁵. We assume that laminar neurons in an auditory specialist like the barn owl are twice as fast as those in chicken and model e.p.s.ps with a width of 250 μ s (Fig. 1a, inset).

We concentrate on a single-frequency channel and stimulate with a pure tone of, for example, 5 kHz (period $T = 200 \mu$ s). The input from magnocellular neurons to our 'integrate-and-fire' model neuron in the laminar nucleus is described as a sequence of spikes (Fig. 1b). Presynaptic spikes occur preferentially around a specific mean phase of the tone¹³. A 'jitter' of 40 μ s models internal sources of variability and noise along the auditory pathway as well as the bandwidth of frequency tuning of auditory neurons. The mean firing rate is relatively low (667 Hz) so that each presynaptic spike train skips most of the cycles of the 5 kHz tone.

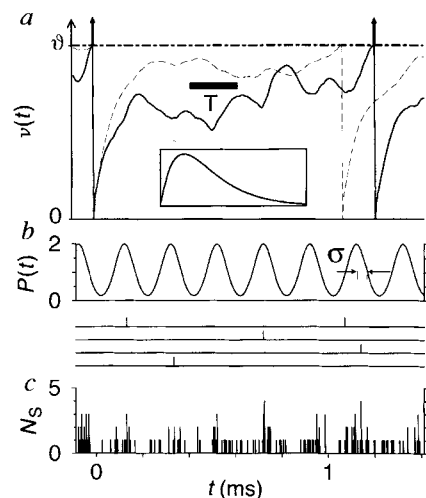


FIG. 1 a, Membrane potential v of an integrate-and-fire neuron as a function of time. b, Probability density $P(t)$ of presynaptic firing during 5-kHz stimulation and four samples of input spike trains (vertical bars). The model neuron receives input from 154 presynaptic neurons^{1,19} in volleys of phase-locked spikes with a 'jitter' of 40 μ s driven by a 5-kHz tone. Spikes are generated by a stochastic process with periodically modulated rate (solid line in b). A histogram of spike arrival times (number of spikes N_s in bins of 5 μ s) summed over all 154 synapses is shown in c. Each input spike evokes an e.p.s.p. shown on an enlarged voltage scale (same time scale) in the inset of a. The e.p.s.ps from all neurons are added linearly and yield the membrane voltage v (a, main figure). With the spike input shown in c the membrane voltage exhibits oscillations (solid line). The model neuron fires (arrow), if v reaches a threshold v . Firing must always occur during the time when v increases so that, in the case of coherent input, output spikes are phase locked as well. If input spikes arrive incoherently, v follows a trajectory with statistical fluctuations, but no systematic oscillations (dashed line) and the output spikes are not phase locked. Time scales in a–c are identical; the period $T = 200 \mu$ s is indicated by a horizontal bar. Voltage in a: arbitrary units; the threshold v is 36 times the amplitude of a single e.p.s.p. Rate in b in kHz.

METHODS. The differential equation $dv/dt = -v/\tau_m + I(t)$ has been integrated (time step 5 μ s) with an effective membrane time constant $\tau_m = 100 \mu$ s. An input spike arriving at time t_j^i at a synapse j evokes an input current $I_j^i(t) = (1/\tau_s) \exp(-(t - t_j^i)/\tau_s)$ for $t < t_j^i$ with $\tau_s = 100 \mu$ s. The total input is $I(t) = \sum_j J_j I_j^i(t)$ where J_j is the efficacy of synapse j and the sum runs over all synapses and firing times preceding t . For each input channel j , firing times are generated with probability density $P(t) = (\pi T/\sigma\sqrt{2\pi}) \sum_{m=-\infty}^{\infty} \exp[-(t - mT - \Delta_j)^2/2\sigma^2]$ where $\pi = 1$ kHz is a rate, $T = 200 \mu$ s is the period of the stimulation tone, Δ_j is the transmission delay from the ear to the laminar nucleus, and $\sigma = 40 \mu$ s is the temporal jitter. We impose absolute refractoriness through a minimal interspike interval of 0.5 ms. For incoherent spike arrival (dashed line in a), the transmission delays Δ_j are drawn from the broad distribution of Fig. 2a, for coherent spike arrival (solid line in a) the delay distribution is that of Fig. 2c. To define the effective time constant τ_m of the integrate-and-fire model, we started from a more detailed model with an explicit description of the outward rectifying current^{14,15}. See Supplementary Information for details.

Spikes from more than 100 presynaptic neurons (Fig. 1c) produce e.p.s.ps that arrive coherently with a common mean phase and lead to an oscillating membrane potential v . The membrane potential builds up, reaches the threshold in the rising stage of the cycle and in this way produces slightly more accurate phase locking than that of the input (Fig. 1a). If presynaptic spikes arrive with random phases, however, v shows aperiodic fluctuations (Fig. 1a), and the output spikes have a uniform phase distribution (Fig. 2a). We expect this to be the initial, embryonic condition before the connections between the magnocellular and the laminar nucleus are tuned during a sensitive period¹⁸. In adult owls, transmission delays from the ear to the laminar nucleus differ greatly. Their mean value is between 2 and 3 ms, and the standard deviation is about 200–240 μ s (ref. 19). Without tuning of the delays, any phase information would be lost.

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FIG. 2 Hebbian learning. In *a*–*c*, the left-hand graph shows synapses binned as a function of the signal transmission delay Δ . On the right-hand side, the distributions of output phases are shown in period histograms (bin width 5 μ s). *a*, Before learning, there are 600 synapses with a broad distribution of signal transmission delays (left) drawn from a gaussian distribution (2.5 ± 0.3 ms). The output of the laminar model neuron shows no phase locking to a 2-kHz (not shown) or a 5-kHz signal (right). *b*, After a learning session while being stimulated by a 2-kHz signal. The 105 synapses that survive learning have delays which differ by multiples of the period $T = 500 \mu$ s (scale bar). The output spikes exhibit phase locking with vector strength²⁵ $v = 0.97$ corresponding to a temporal precision of 20 μ s (right). *c*, Same plot as in *b*, after learning a 5-kHz input signal. 154 saturated synapses survive. The output spikes exhibit phase locking ($v = 0.75$) corresponding to a temporal precision of 25 μ s. *d*, Learning window W as a function of the delay s between postsynaptic firing and presynaptic spike arrival. The graph on the right-hand side shows the boxed region around the maximum on an expanded scale. If $W(s)$ is positive (negative) for some s , the synaptic efficacy is increased (decreased). The postsynaptic firing occurs at $s = 0$ (vertical dashed line). Learning is most efficient if presynaptic spikes arrive shortly before the postsynaptic neuron starts firing as in synapse A. Another synapse B which fires after the postsynaptic spike is weakened.

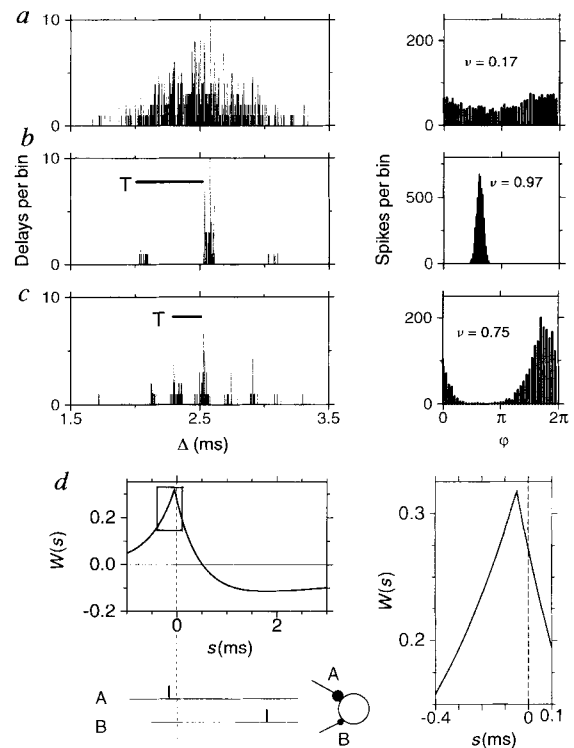
METHODS. Before learning, all synapses have identical efficacies $J_i = 1$. During learning, efficacies are changed according to $\Delta J_i = \epsilon \sum_r [\gamma + \sum_n W(t_i^r - t_n^r)]$ with factors $\epsilon = 0.002$, $\gamma = 0.1$. The sum runs over all spike arrival times $t_i^r < t$ and all postsynaptic firings $t_n^r < t$. We take $W(s) = 0.3 \exp[(s + 0.05)/0.5]$ for $s < -0.05$ and $W(s) = 0.5 \exp[-(s + 0.05)/0.5] - 0.2 \exp[-(s + 0.05)/5]$ for $s \geq -0.05$ where s is a time in ms. Learning occurs during 3,000 seconds in time steps of 5 μ s. The synaptic strength saturates at a maximum of 3. A synapse whose efficacy vanishes is removed. The efficiency of phase locking is quantified by the vector strength²⁵. Spike input and electrical time constants are as in Fig. 1.

A hebbian learning rule proves to be an efficient tuning mechanism.

In hebbian learning, a synaptic efficacy is changed by a small amount ϵ , if presynaptic spike arrival and postsynaptic firing coincide. This simultaneity constraint is implemented by a learning window $W(s)$ where s is the difference between the arrival time of a presynaptic spike and the postsynaptic firing. In our model, $W(s)$ has two regimes (Fig. 2*d*). For $s < 0$, $W(s)$ is positive. Thus, the efficacy of synapses which are repeatedly active shortly before a postsynaptic spike occurs, is increased^{20–22}. The efficacy of synapses which are active shortly after the postsynaptic spike is decreased^{23,24}. As depolarization is known to induce potentiation of active synapses²² and as the neuron is depolarized most of the time between two spikes, we also strengthen each active synapse by a small amount γ , even if no postsynaptic spike occurs. The procedure of continuously strengthening and weakening the synapses automatically leads to a normalization of the total input strength to the postsynaptic neuron in a competitive self-organized process.

As a result of learning, a clearly structured distribution of synapses evolves (Fig. 2*b, c*). Delays of the remaining synapses differ roughly by multiples of the period T of the stimulating tone. Before learning, the cell was not tuned to this period. The final synaptic pattern of a neuron stimulated by a 2-kHz signal during learning is different from that of a 5-kHz neuron (Fig. 2*b, c*).

The evolution of synaptic efficacy during learning does not depend on the specific shape of the learning window W but only on some generic properties. In particular, the learning window can extend over several milliseconds²⁴ and therefore is large compared with the period T of the sound (Fig. 2*d*). Efficient learning depends on the temporal relationship between the process of strengthening the synaptic efficacies and that of weakening them. The maximum of the window function $W(s)$ should be at a location $\tilde{s} \approx -\tau_s/2$ where τ_s is the rise time of the postsynaptic potential (Fig. 2*d*). This can be understood as follows. Let us assume that learning has led to a sharply peaked distribution with all synapses having a common delay Δ (modulo T). Coherent input arriving at the synapses can trigger a postsynaptic spike with a mean delay of roughly $\tau_s/2$. Because of learning, all synapses which are active



slightly before the postsynaptic firing will be strengthened. If $\tilde{s} = -\tau_s/2$, the maximal increase of synaptic efficacy occurs at those synapses which are already strongest.

So far we have considered monoaural input, but laminar neurons also exhibit a sensitivity to the interaural time difference (ITD)^{19,25,26}. We divide the synapses into two groups, with input from the left or the right ear. During learning, both ears are stimulated by the same signal and with a fixed ITD. The learning rule selects synaptic connections so that spikes arrive coherently for exactly this ITD. If the same ITD is used after learning, the neuron is driven optimally and emits phase-lock spikes. If the ITD

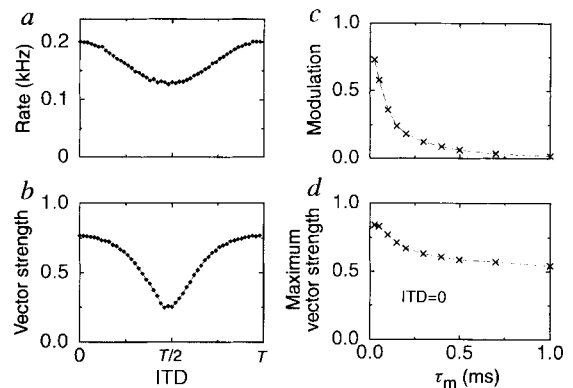


FIG. 3 *a, b*, Turning to interaural time difference (ITD). The output rate (*a*) and the vector strength (*b*) of a laminar neuron are shown as a function of ITD. The model neuron has been tuned to a 5 kHz signal ($T = 200 \mu$ s) as described in the text and the legend of Fig. 2. Half of the 154 synapses which survive learning transmit signals from the left ear, the others from the right ear. The neuron exhibits best phase locking and maximal output rate $f_{\max}$ for the interaural delay used during learning (ITD = 0). The rate has a minimum $f_{\min}$ for ITD = $T/2$. *c, d*, Effect of τ_m . We define the modulation as $(f_{\max} - f_{\min})/f_{\max}$ and plot the modulation as a function of the effective membrane time constant τ_m in *c*. The vector strength at ITD = 0 is shown as a function of τ_m in *d*.

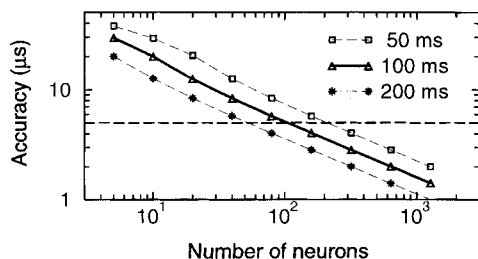


FIG. 4 Population coding. The temporal precision that can be achieved in a population of N independent neurons is shown as a function of N under the assumption of population vector coding^{27,28}. The ITD is estimated based on spikes occurring in a time window of $\tau = 50$ ms (squares), 100 ms (triangles), or 200 ms (stars). The accuracy is defined as the average deviation of the estimate from the actual ITD of the stimulus. The horizontal line at 5 μ s is the boundary given by the behavioural performance of 1–2° of azimuthal angle¹². For large N , the accuracy scales as $1/\sqrt{\tau N}$ as expected from the central limit theorem.

METHODS. We took N neurons $1 \leq k \leq N$ with ITD tuning curves similar to Fig. 3a, approximated by $f_k(x) = 164 + 36 \cos[(x - x_k)2\pi/T]$ Hz where x is the ITD and T the period of the stimulation, x_k is the optimal ITD of neuron k and f_k its mean firing rate in Hz. The values of x_k have a uniform distribution between 0 and T . Spikes of neuron k are generated by a Poisson process with mean rate f_k . In an additional simulation (data not shown) we have confirmed that Poisson statistics give a reasonable assumption and these results do not change, if we generate spikes by a model neuron as in Fig. 1. The ITD of the stimulus is estimated as $x^{\text{est}} = (T/2\pi) \arg(\sum_k n_k e^{i2\pi x_k/T})$ where n_k is the number of spikes of neuron k measured in a time-window of length τ . We plot the accuracy $\langle |x^{\text{est}} - x|^2 \rangle^{1/2}$ where the angular brackets denote an average over 20,000 trials.

does not match, phase locking of the output spikes breaks down and the mean firing rate decreases (Fig. 3a, b).

Temporal information conveyed by a single laminar neuron is limited. The temporal precision of phase locking is about 20–25 μ s (Fig. 2) and the ITD tuning curve is only weakly modulated (Fig. 3a). Nevertheless, barn owls achieve a behavioural performance corresponding to a temporal precision of 5 μ s. The barn owl has a reaction time of about 100 ms before it initiates a head movement¹². During 100 ms the firing pattern of a population of laminar neurons potentially contains enough information to resolve ITDs with a precision of 5 μ s.

We consider a group of laminar neurons with ITD tuning curves as in Fig. 3a, but shifted along the ITD axis so that the optimal responses occur at different ITDs. Neurons are stimulated by a tone with a fixed, but unknown, ITD. We estimate the ITD from the neuronal firing pattern by a 'population vector' decoding scheme^{27,28}: the ITD corresponding to the optimal response of each neuron is noted, and each value is weighted according to the number of spikes that occur in a time window of 100 ms. We find that the activity of about 100 independent neurons provides enough information to estimate the ITD with a precision of 5 μ s (Fig. 4) apart from ambiguities that are caused by periodicity of the signal. Weak correlations from common inputs rescale the absolute values but do not alter the conclusions.

The firing precision of 20–25 μ s of single neurons has been achieved despite the fact that input spikes evoke e.p.s.p.s which are ten times broader. In an additional set of simulations, we have systematically varied the effective membrane time constant τ_m and hence the width of the e.p.s.p. We find that the temporal precision of neuronal spiking depends only weakly on τ_m (Fig. 3d). This is possible because firing always occurs during the rise time of a postsynaptic potential. On the other hand, for a 5-kHz signal, ITD tuning breaks down rapidly, if τ_m exceeds 0.1 ms (Fig. 3c). Thus modulation of ITD tuning does indeed require short e.p.s.p.s. In fact, very short time constants have been measured^{14–15}.

Our results show that the temporal precision of output spikes need not be limited by the membrane time constant. Spike timing can achieve a resolution shorter than the rise time of an e.p.s.p. given coherent spike arrival; and spike arrival times can be tuned

accurately by a hebbian learning rule. It is tempting to apply the same ideas to information processing in the hippocampus⁵, the cerebellum²⁹, or the cortex⁵. If we increase time constants by a factor of about 100 and reinterpret our results, then we are led to the conclusion that in areas where effective membrane time constants are in the range order of 10–20 ms (ref. 30), a temporal code with an accuracy of 1–3 ms would be possible. □

Received 24 April; accepted 4 July 1996.

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ACKNOWLEDGEMENTS. We thank D. Kautz, C. Köppl, and A. Reyes for helpful discussions, and R. G. Palmer for critical reading of the manuscript. W.G. and R.K. were supported by the DFG.

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Thalamic modulation of high-frequency oscillating potentials in auditory cortex

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PERHAPS the most widely recognized but least understood electrophysiological activity of the cerebral cortex is its characteristic electrical oscillations. Recently, there have been efforts to understand the mechanisms underlying high-frequency gamma oscillations (~40 Hz) because they may coordinate sensory processing between populations of cortical cells^{1,2}. High-resolution cortical recordings show that gamma oscillations are constrained to sensory cortex^{3–5}, that they occur independently in auditory and somatosensory cortex⁴, and that they are phase-locked between primary and secondary sensory cortex⁵. As yet, the mechanism of their neurogenesis is unknown². Whereas cortical neurons can produce gamma oscillations without subcortical input^{6–9}, they may also be modulated by the thalamus¹⁰ and basal forebrain¹¹. Here we report that the neural generator of gamma oscillations in

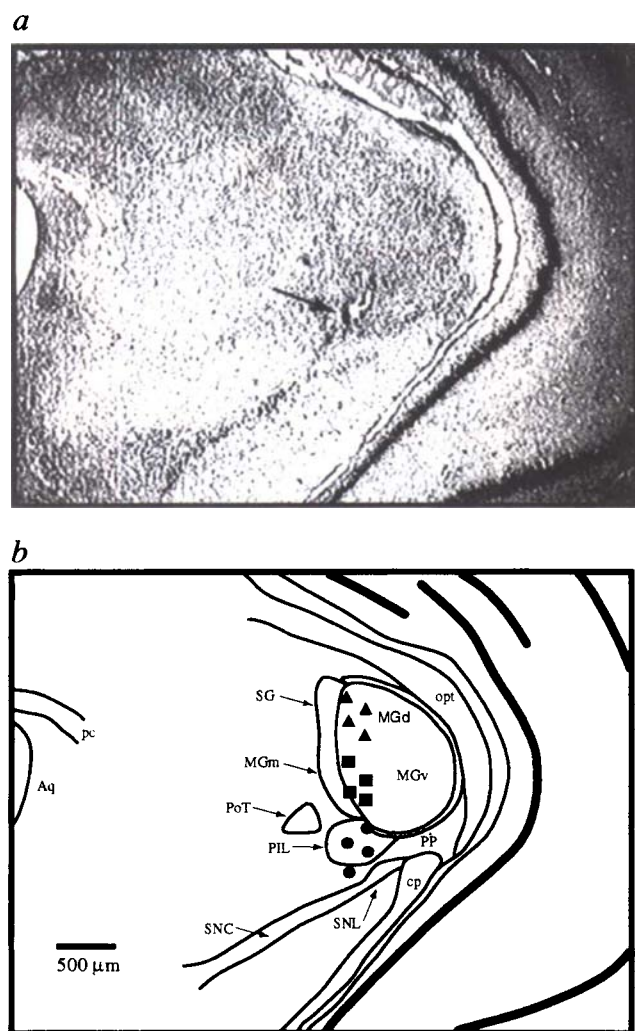


FIG. 1 Electrical stimuli were delivered to subdivisions of the acoustic thalamus in the right hemisphere with a bipolar stainless steel electrode (RMI model NE-200), lowered in 100- μ m increments along a dorsoventral track, 5 mm posterior to the bregma and 3-mm lateral to the midline. *a*, A marking lesion (arrow) was produced (5 mA, 10 s) in the most ventral location capable of producing gamma oscillations in auditory cortex. *b*, Stimulation sites in four animals within the dorsal (triangles) and ventral (squares) divisions of the medial geniculate nucleus, and the posterior intralaminar nucleus (circles). The identity and approximate borders of structures were adapted from ref. 23. MGd, medial geniculate dorsal; MGv, medial geniculate ventral; MGm, medial geniculate medial; SG, supragenicular; PIL, posterior intralaminar; PoT, posterior triangular; PP, peripeduncular; SNL, substantia nigra lateral; SNC, substantia nigra compact; cp, cerebral peduncle basal; opt, optic tract; Aq, aqueduct; pc, posterior commissure.

auditory cortex seems to be intracortical, serving to synchronize interactions between the primary and secondary areas. The acoustic thalamus directly modulates these oscillations, which are inhibited by stimulation of the dorsal and ventral divisions of the medial geniculate nucleus (MGd and MGv) and evoked by stimulation of the adjacent posterior intralaminar nucleus (PIL).

Regions of the acoustic thalamus were electrically stimulated with 0.5-s trains of current pulses and resulting potentials recorded from the surface of auditory cortex in lightly halothane-anaesthetized rats. When stimuli were delivered to the right MGd and MGv (Fig. 1), the cortical response consisted of a biphasic positive-negative wave (Fig. 2*a, b*), followed by a large-amplitude positive slow potential. Movement of the stimulating electrode 300 μ m ventral to the MGv into the PIL (Fig. 1*b*), resulted in a marked change in the cortical response: the initial

fast wave became monophasic and negative and the slow potential was replaced by oscillations in the gamma-frequency band (Fig. 2*c, d*).

Maps of the slow potentials, recorded from a square 64-electrode array positioned over the primary and secondary auditory cortex in the right hemisphere (Fig. 2*e*), indicate that they were centred on the secondary auditory cortex (area 36) during MGd stimulation, shifted to the primary auditory cortex (area 41) during stimulation of the MGv, but were replaced by gamma oscillations centred between primary and secondary auditory cortex during stimulation of the PIL (Fig. 2*f*). The region of the PIL capable of evoking gamma oscillations was quite small. Movement of the stimulating electrode more than 500 μ m in any direction resulted in loss of the cortical response. However, whereas the effective thalamic zone seemed to be constrained to the PIL, fibres of passage may also have contributed to the evoked gamma response and cannot be ruled out.

Without stimulation, spontaneous cortical activity was frequently punctuated with bursts of gamma oscillations having a focal and consistent spatial pattern (Fig. 3*a*). The frequency, power, and surface distribution of these bursts were quite similar to activity evoked by PIL stimulation (Fig. 3*b*, left). Surprisingly, electrolytic lesions sufficient to completely eliminate evoked gamma oscillations had no apparent effect on the spontaneous bursts (Fig. 3*b*, right). Similarities in evoked and spontaneous gamma oscillations were also demonstrated using phase-locked averaging of successive waves (Fig. 4). Gamma waves in the secondary auditory cortex (Fig. 4*a*) were systematically delayed by 2–4 ms compared to those in the primary auditory cortex (Fig. 4*b*) during both spontaneous and evoked bursts.

These results indicate that the anatomical subdivisions of acoustic thalamus play functionally distinct roles in activating auditory cortex. The MGv and MGd are the principal relay nuclei for auditory input to the cortex. The finding that primary and secondary auditory cortex selectively respond to stimulation of the MGv and MGd concurs with the established parallel thalamocortical projections of these subnuclei^{12,13}. Although the thalamic relay nuclei were not initially thought to participate in the genesis or synchronization of cortical fast rhythms, recent evidence indicates that they may produce gamma oscillations in synchrony with cortical areas with which they are reciprocally connected¹⁰. However, medial geniculate stimulation did not evoke gamma oscillations in the auditory cortex of any of our animals, and if the stimulus train occurred during a spontaneous burst, the burst was promptly aborted. Our data indicate that the sensory relay nuclei of the thalamus are not involved in the generation of cortical gamma oscillations and may actually interrupt the neural oscillator when sufficiently activated.

In contrast, stimulation of the PIL always produced gamma oscillations in auditory cortex. Whereas the PIL is clearly part of the acoustic thalamus in the rat^{14–16}, its function is poorly understood. The anterior intralaminar nuclei, specifically the centrolateral nucleus, have recently been implicated in the production of cortical gamma oscillations in cat sensorimotor cortex^{10,17}. Simultaneous recordings from the cortex and centrolateral nucleus reveal closely correlated fast rhythms, indicating that cortical and intralaminar thalamic neurons may become synchronized, and that thalamocortical pathways may be involved in the genesis of gamma oscillations. Our results support anatomical studies suggesting that the PIL constitutes a posterior extension of the intralaminar complex^{18,19}, producing gamma oscillations in the auditory cortex. Yet, the close similarity between the spatial and temporal properties of oscillations evoked by PIL stimulation and those of spontaneous gamma bursts, and the fact that lesions of the PIL sufficient to abolish evoked gamma oscillations had no detectable influence on spontaneous bursts, suggests that the neural oscillator is intracortical and can function independently of subcortical input. This conclusion is supported by evidence that both layer V pyramidal cells⁸ and layer IV inhibitory interneurons⁷ can produce subthreshold oscillations in the gamma frequency

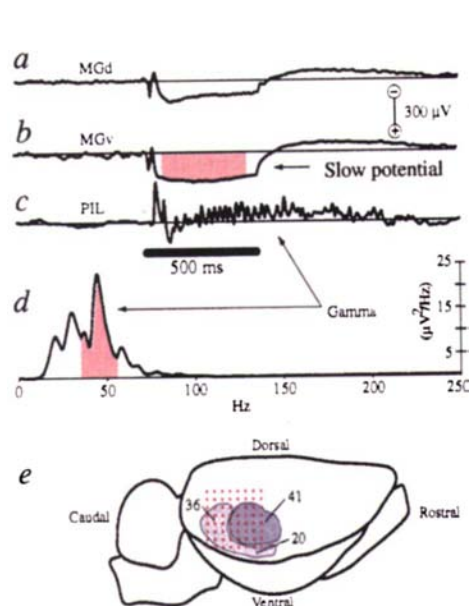
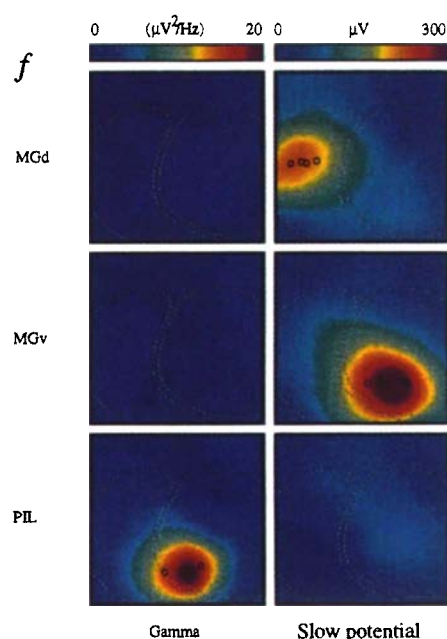


FIG. 2 Surface potentials evoked in the auditory cortex during electrical stimulation of the acoustic thalamus. *a*, 500-ms trains of current pulses ($10\ \mu\text{A}$; $0.5\ \text{ms}$ duration; $500\ \text{Hz}$) delivered to the MGd produced a positive-negative fast wave followed by a prolonged positive slow potential for the duration of the stimulus. *b*, A similar response was evoked by stimulation of the MGv. The slow potential was quantified for subsequent mapping by averaging its amplitude from 100 to 400 ms post-stimulus (shaded area). *c*, Stimulation of the PIL resulted in a response that began with a negative fast wave followed by rhythmic gamma oscillations often continuing for roughly 100 ms after termination of the stimulus train. *d*, Power spectral density of the evoked oscillations between 100 and 400 ms post-stimulus peaked near 40 Hz, in the gamma frequency band. The magnitude of gamma oscillations was quantified for subsequent mapping by averaging the power between 35 and 55 Hz (shaded area). *e*, Evoked surface potentials were mapped from an 8×8 electrode array covering a $3.5 \times 3.5\ \text{mm}^2$ area of primary (area 41; dark shaded region) and secondary (area 36 and area 20; light shaded region) auditory cortex in the right



hemisphere. Responses from all 64 electrodes were simultaneously amplified (bandpass cutoff $\pm 6\ \text{dB}$ at $0.01\text{--}100\ \text{Hz}$, roll-off = $5\ \text{dB}$ per octave) and digitally sampled ($500\ \text{Hz}$). *f*, Maps of power in the gamma-frequency band (left column) and slow-potential amplitude (right column) averaged across animals for the three different stimulation conditions. The white dotted lines on each map correspond to the outlines of the primary and secondary auditory cortex. Stimulation of the MGd and MGv resulted in no gamma oscillations, but a prominent slow potential was centred in the secondary and primary auditory cortex, respectively. In contrast, stimulation of the PIL did not produce a slow potential, but instead evoked a focus of gamma oscillations overlapping the boundary between primary and secondary auditory cortex. The peak locations of these maps for the individual animals are superimposed as circles. Colour bars represent $0\text{--}20\ \mu\text{V}^2/\text{Hz}$ averaged spectral power between $35\text{--}55\ \text{Hz}$ for gamma and $0\text{--}300\ \mu\text{V}$ for slow potentials.



FIG. 3 Spontaneous gamma bursts (2 min) were digitized immediately before and 10 min after electrolytic lesioning ($25\ \text{mA}$, $10\ \text{s}$) of the PIL. *a*, A 1-s sample of ongoing electrocortical oscillations recorded from the 64-channel surface array. Gamma bursts (shaded strips) were visually identified on a computer graphics screen and resampled in 128-ms blocks for power spectrum estimation. *b*, The surface distribution of power in the gamma frequency band for the spontaneous bursts was quite similar to bursts evoked by PIL stimulation, with a focus centred on the caudal and lateral border between the primary and secondary auditory cortex (outlined in white dotted lines). Maps of spontaneous gamma power averaged across two animals before (left map) and after (right map) electrolytic lesioning of the PIL ($\sim 1\ \text{mm}$ in diameter and sufficient to eliminate evoked gamma oscillations completely) were nearly identical, suggesting that the gamma oscillator is intracortical and can function independently of PIL input.

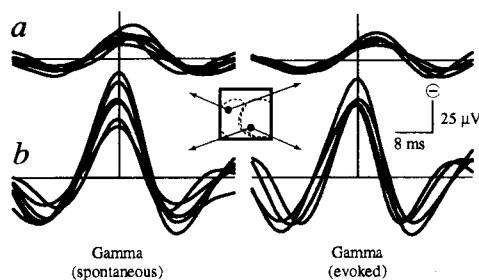


FIG. 4 To obtain a more detailed comparison of the spatiotemporal characteristics of spontaneous and evoked gamma oscillations, a method of phase-locked averaging was devised. An electrode over the primary auditory cortex that exhibited the highest spectral power in the gamma frequency band, was chosen as a temporal reference for averaging of successive gamma waves in all 64 electrodes. Averaging was phase-locked to the negative peaks of gamma waves ($N \approx 300$ for each animal) at this electrode site using an automated peak-seeking algorithm. In the secondary auditory cortex (a), both spontaneous gamma bursts (left traces; superimposed averages of 6 animals) and bursts evoked by PIL stimulation (right traces; superimposed averages of 4 animals) were systematically delayed by 2–4 ms compared with similar activity in the primary auditory cortex (b). The inset indicates the electrode sites plotted. Averaging was time-locked to the peaks of gamma oscillations in the primary auditory cortex.

band. Thus, individual cortical cells and/or small interconnected cell assemblies in the auditory cortex may serve as the neural generator of gamma oscillations, whereas modulation results from input of the PIL.

It has been proposed that synchronized, high-frequency oscillations in the gamma band may be fundamental in the functional linking or coordination of information processing in separate neuronal populations within and between cerebral hemispheres, within the sensory cortex, and even within and between single cortical columns^{1,2,20}. Our results, obtained with high-resolution surface mapping of the auditory cortex, extend earlier results from the auditory and somatosensory cortex^{4,5}, indicating that both evoked and spontaneous gamma bursts represent an asynchronous but tightly phase-locked oscillation between primary and secondary cortex for a given sensory modality. The fact that averaged gamma waves in the secondary cortex are consistently delayed in relation to those in the primary cortex suggests a one-way propagation of activity between the primary and secondary zones through intracortical connections²¹. Thus, whereas the MGv and MGd send parallel thalamocortical projections to^{12,13}, and may independently activate²², primary and secondary auditory cortex, respectively, gamma oscillations modulated by the PIL may reflect coupling of sensory processing between these cortical zones, effected by corticocortical pathways. □

Received 22 April; accepted 3 July 1996.

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ACKNOWLEDGEMENTS. This research was supported by the United States Public Health Service, the NSF, a Grant in Aid from the Graduate School Council on Research and Creative Work at the University of Colorado at Boulder, and an undergraduate research opportunity award from the University of Colorado at Boulder to K.D.M.

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Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex

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THE normal development of T cells in the thymus requires both positive and negative selection. During positive selection, thymocytes mature only if their T-cell receptors react with some specificity to host major histocompatibility complex (MHC) and host peptides. During negative selection, thymocytes die if their T-cell receptors react with too high an affinity to the presenting cell, self MHC, and peptides to which they are exposed. These two processes are important for the development of the T-cell repertoire and the acquisition of self-tolerance, but their precise location and temporal relationship are not known. We have used the keratin 14 (K14) promoter to re-express a class II MHC antigen (I-A^b) in class II-negative mice. The transgenic I-A molecule is expressed only on thymic cortical epithelium; thymic medullary epithelium and bone-marrow-derived cells are I-A negative. CD4⁺ cells are positively selected in K14 mice, but clonal deletion does not occur in K14 mice or in reB-negative mice, which lack a thymic medulla. The K14 CD4 cells are autoreactive, as they proliferate extensively to and specifically lyse I-A^b-positive target cells. These autoreactive cells make up 5% of the peripheral CD4 T cells, providing an estimate of the minimal frequency of positively selected cells that must subsequently undergo negative selection for self-tolerance to be preserved. Thus positive and negative selection occur in anatomically distinct sites.

The A_β gene was reintroduced into C57Bl/6 class II-negative $A_\beta^{-/-}$ mice by using a complementary DNA transgene controlled by the human K14 promoter¹, which targets transgenes to stratified squamous epithelium. Expression of mature I-A heterodimers is expected to be limited to those tissues in which transgenic A_β expression overlaps with endogenous A_β expression. Thus we found that keratinocytes and other stratified squamous epithelia are I-A negative in these K14 (K14) transgenic mice (data not shown). Immunohistochemistry of the thymus showed that expression of I-A is restricted to thymic cortical epithelium (Fig. 1c, d) as there is no overlap in staining between M5/114 (anti-class II) and UEA-1, a lectin that stains thymic medullary epithelium. In addition, flow cytometric analysis of splenic B cells, macrophages and dendritic cells demonstrates no I-A expression on cells of haematopoietic origin (Fig. 1f–h). This expression pattern is distinct from a previously described class II I-E transgenic, 36-5 (ref. 2), in which peripheral cells were I-E negative but both cortical and medullary epithelium expressed I-E (Fig. 1e). It

is also distinct from the pattern of transgene expression in the *I-E* transgenics ΔX and ΔY , which express E_α on both bone-marrow-derived and thymic epithelial cells³. The tissue distribution is identical in all K14 lines examined (Fig. 1). Multiple transgenic founder lines were generated by using two different A_β cDNAs: the endogenous A_β gene, or a cDNA in which the first 47 amino acids of the A_β cDNA are of the k haplotype (kbb), a molecule serologically indistinguishable from wild-type *I-A^b* (ref. 4). Although the level of *I-A^b* expression detected by immunohistochemistry seems to be significantly lower than that in either K14/*I-A^b* or wild-type mice, similar results were obtained for all K14 lines in the experiments described below.

Flow cytometry established that peripheral lymphocyte populations in the K14 mice contain a substantial number of CD4 T cells (ranging in various founder lines from 5% to 18% of total splenocytes), as compared with the class II-negative mice, which have 1% to 4% CD4⁺ cells⁵. Moreover, unlike the few CD4⁺ cells in the periphery of class II-negative mice⁶, the pattern of T-cell antigen receptor (TCR) V_β usage by the K14 CD4⁺ cells is the same as that of A_β (+/-) littermate controls (not shown). In addition, analysis of the thymocyte maturation markers, CD44, CD69, CD25, heat-stable antigen and Mel14 suggests that thy-

mocyte-positive development is temporally similar in the K14 and wild-type thymi (data not shown). Thus targeting of A_β expression to thymic cortical epithelium rescues the positive selection of CD4⁺ cells.

To test whether CD4⁺ T cells selected on cortical epithelial class II molecules are functional, mixed lymphocyte responses (MLRs) were examined. Splenocytes from K14 mice, like those of wild-type mice, proliferated extensively in response to irradiated allogeneic stimulator cells (Fig. 2a). However, K14 transgenic splenocytes also exhibited extensive proliferation to *I-A^b*-positive C57Bl/6 stimulator cells (Fig. 2a, b and data not shown). Notably, the autoreactive response was greater than the allogeneic response. K14 thymocytes also proliferated in response to *I-A^b* stimulators (Fig. 2c).

The significant autoreactivity of the K14 CD4⁺ T cells could be a consequence of the absence of *I-A* expression on either peripheral antigen-presenting cells (APCs) or on medullary epithelium. The reactivity of K14 thymocytes to *I-A^b* stimulators suggests that the self-reactive phenotype develops in the thymus, rather than the periphery. To confirm this, we examined the proliferative response in MLRs of splenocytes from 36-5 E_α transgenic mice (which express wild-type patterns of *I-E* on both

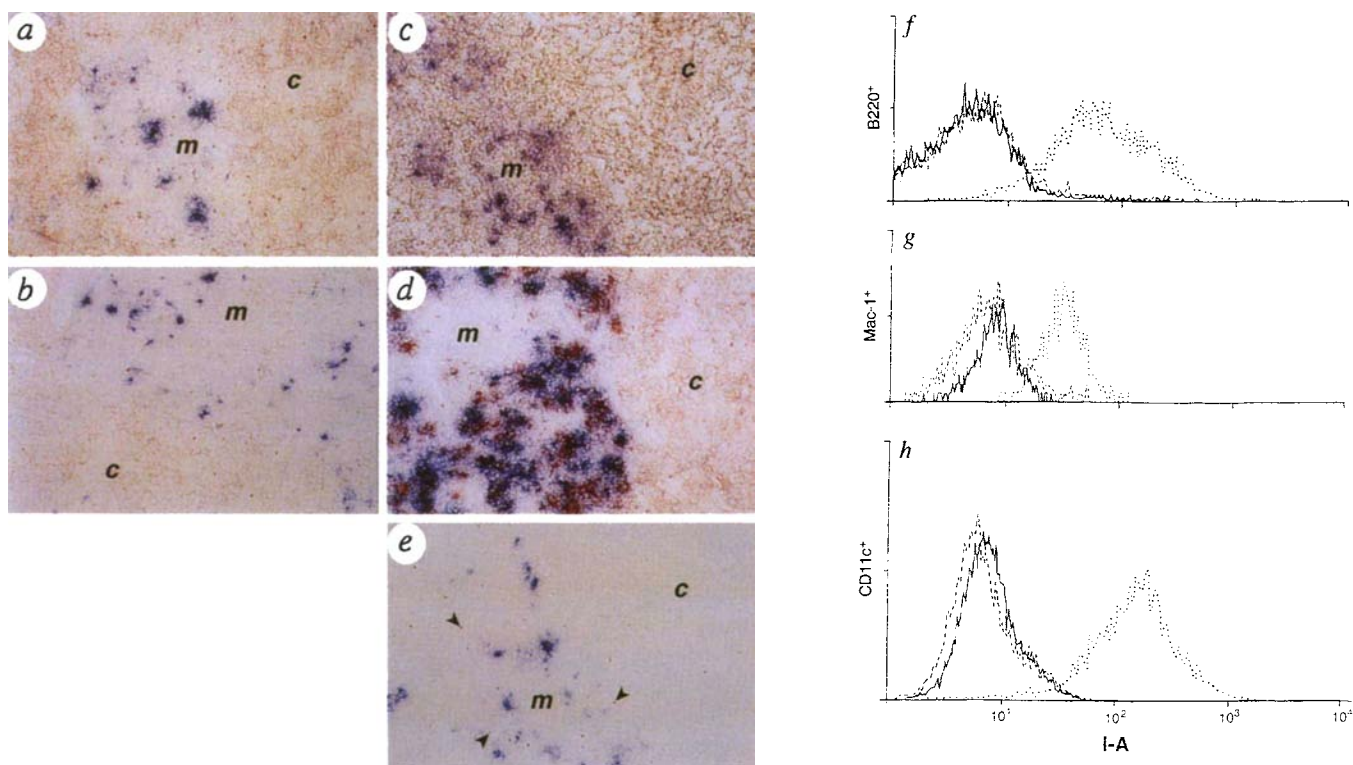


FIG. 1 Production and characterization of K14 transgenic mice. a-e, Histological analysis of thymic tissues from transgenic and control mice. Thymus sections were stained for MHC class II (red) and the medullary epithelial cell marker UEA-1 (blue). The medullary regions (m) and cortical regions (c) are identified. a, K14/*I-A^{kbb}* thymus, showing expression of class II transgene exclusively in cortical epithelium; b, K14/*I-A^{kbb}* thymus with weaker class II expression, but pattern identical to a; c, A_β (+/-) thymus showing normal cortical and medullary class II staining pattern. Note that all UEA-1-positive cells are also class II positive; d, transgenic mouse line 36-5, which expresses class II *I-E* on cortical and medullary epithelium, but not on bone-marrow-derived elements; medullary class II expression colocalizes exclusively with UEA-1 staining and not with dendritic cells. e, A_β (-/-) thymus showing absence of class II staining, but normal UEA-1 staining. Arrowheads identify the granular staining of thymic medullary macrophages and granulocytes, which express endogenous peroxidase activity, and account for the granular red staining in the medulla of the other thymus sections. f-h, Flow cytometric analysis of *I-A^b* splenic B cells (f), interferon (IFN)- γ -treated splenic macrophages (g), and dendritic cells treated with granulocyte macrophage colony-stimulating factor (GM-CSF) (h), from A_β

(-/-) (dashed line), A_β (+/-) (dotted line), or K14/*I-A^{kbb}* mice; this demonstrates that K14 APCs are class II negative.

METHODS. A_β cDNAs were ligated into the *Bam*HI site of a transgene vector¹ containing 2 kb of the human K14 promoter 5' of the cloning site, and most of the human growth hormone gene and polyadenylation signal 3' of the cloning site. A 5-kb *Kpn*I-*Xba*I fragment of DNA was injected into the pronucleus of fertilized C57Bl/6 eggs. Transgenic founders were backcrossed eight (kbb) or thirteen (bbb) generations to C57Bl/6 A_β (-/-) mice. Immunohistochemistry (a-e) was performed as previously described⁷, using the antibodies M5/114 (anti-*I-A^b*) or 14-4-4s (anti-*I-E*). B cells were stained with PE-labelled RA3-6B2 (anti-CD45 (B220), Pharmingen) and fluorescein isothiocyanate (FITC)-labelled AF6-120.1 (anti-*I-A^b*, Pharmingen). Splenic adherent cells, cultured for 72 h with 10 U ml⁻¹ IFN- γ were stained with PE-labelled M1/70 (anti-CD11b (Mac-1), Pharmingen) and FITC-labelled AF6-120.1. Splenocytes were cultured in 30 U ml⁻¹ GM-CSF for 24 h. At 24 h, non-adherent cells were stained with PE-labelled HL3 (anti-CD11c, Pharmingen), an antibody specific for dendritic cells and FITC-labelled AF6-120.1. Histograms of PE-positive cells are shown.

cortical and medullary epithelium but, like the K14 mice, lack all peripheral class II expression) to cells from syngeneic 107 E₂ transgenic mice² (which express wild-type patterns of I-E on both thymic and peripheral cells). As is the case with K14 mice, the 36-5 E₂ transgenic thymic epithelium expresses lower levels of class II molecules than that of wild-type mice. The 36-5 splenocytes do not exhibit the profound autoreactive response observed with K14 mice (Fig. 2c). Thus CD4⁺ T cells, which mature in a thymus expressing class II on both cortical and medullary epithelium, do not proliferate to APCs expressing the thymic class II I-A^b antigen if they lack expression of class II molecules on their peripheral APCs. In addition, *relB* (-/-) mice⁷, which completely lack a thymic medulla but express levels of class II on thymic cortical epithelium, peripheral B cells and macrophages similar to wild-type mice, have splenocytes that also generate an autoreactive response (not shown). Taken together, these data suggest that the proliferation to class II-positive cells by CD4⁺ K14 cells results from class II I-A^b expression that is limited to thymic cortex, rather than from dysregulation of CD4⁺ cells caused by absence of class II expression in the periphery.

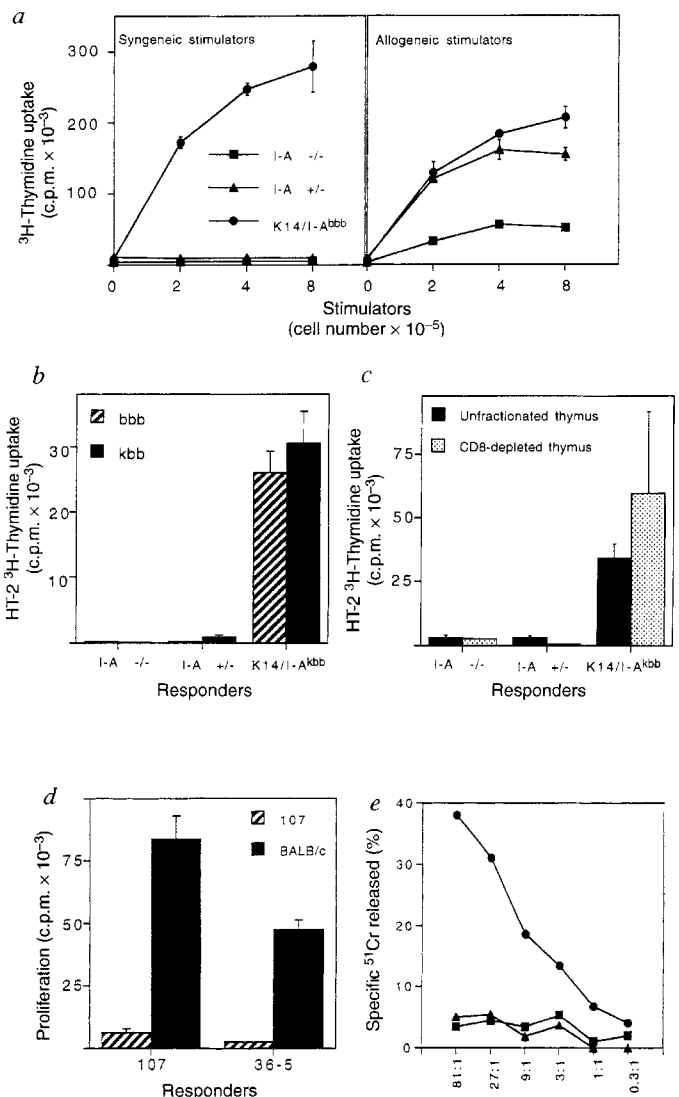
An autoreactive MLR can only be detected in normal mice when specialized APCs are used as stimulator cells⁸. However, in normal mice these self-reactive cells do not lyse syngeneic targets. In contrast, effector cells derived from K14 syngeneic MLR cultures specifically lyse I-A^b-transfected L cell targets (Fig. 2d). Classically, most cytotoxic T cells are class I-restricted CD8⁺ T cells, the development of which should be normal in both K14 and

class II-negative mice. To determine whether K14 CD4⁺ T cells were providing help to autoreactive CD8⁺ effectors or were themselves the cytotoxic effectors, cells collected from K14 MLR cultures were depleted of either CD4⁺ or CD8⁺ cells and cytotoxic activity was assayed. The I-A^b-specific cytotoxic activity segregated with the CD4⁺ population; K14-derived CD8⁺ T cells were not cytotoxic in a ⁵¹Cr release-assay. Thus the CD4⁺ T cells positively selected in the K14 mice both proliferate to and kill cells expressing the thymic class II I-A^b antigen.

To determine the role of thymic cortical epithelium in negative selection, staphylococcal enterotoxin B (SEB) was injected intra-peritoneally into K14 or control, *A_β*^b (+/-) neonatal mice, and V_β TCR usage by mature CD4⁺ T cells was analysed. SEB treatment of neonatal I-A^b-positive mice results in thymic depletion of V_β3⁺ and V_β8⁺ CD4⁺ T cells through clonal deletion⁹. Thus 18–20% of the CD4⁺ thymic T cells in both K14 and *A_β*^b (+/-) phosphate-buffered saline (PBS)-injected controls use a V_β8.1/2-reactive TCR, and SEB injection of wild-type mice resulted in the reduction of V_β8-reactive cells to 5–7% of mature thymic CD4⁺ T cells (Fig. 3a). This decrease was also apparent in CD4⁺ splenocytes (data not shown). However, treatment of K14 neonates with SEB had minimal, if any, effect on the V_β repertoire of thymic CD4⁺ cells (Fig. 3a), demonstrating that thymic cortical epithelium cannot mediate the deletion of SEB-reactive CD4⁺ T cells. Splenocytes from SEB-treated K14 neonates did respond to SEB *in vitro* (data not shown), suggesting that thymic cortical epithelium is not simply tolerogenic.

FIG. 2 T cells and thymocytes from K14 mice proliferate extensively to syngeneic APCs and specifically lyse I-A^b positive target cells. **a**, Responder splenocytes from K14/*I-A^{bbb}* (circles), *A_β*^b (+/-) (triangles) or *A_β*^b (-/-) (squares) were cultured with titrations of irradiated spleen APCs from syngeneic C57Bl/6 or allogeneic BALB/c mice, and ³H-thymidine incorporation was assayed. Only K14/*I-A^{bbb}* splenocytes respond to syngeneic stimulation. **b**, CD8-depleted responder splenocytes (4×10^5) from K14/*I-A^{bbb}*, *A_β*^b (+/-) or *A_β*^b (-/-) mice were cultured with mitomycin C-treated L cells transfected with *A_β*^b and either *A_β*^{bbb} (hatched bars) or *A_β*^{kbb} (black bars), and cytokine production was assayed using the HT-2 indicator cell line. K14/*I-A^{bbb}* splenocytes respond equivalently to both *I-A^{bbb}* and *I-A^{kbb}* stimulators, confirming that the anti-I-A^b proliferation by K14/*I-A^{bbb}* splenocytes was a syngeneic and not an allogeneic response. **c**, Both 4×10^5 unfractionated (black bars) and 3×10^5 CD8-depleted (dotted bars) thymocytes from K14/*I-A^{bbb}* mice produce cytokines when cultured with *A_β*^b/*A_β*^{bbb}-transfected L cells. **d**, Responder splenocytes (4×10^5) from 107 (wild-type I-E) or 36-5 (thymic epithelial I-E) were cultured with 8×10^5 irradiated spleen APCs from 107/I-E (syngeneic, hatched bars) or BALB/c (allogeneic, black bars) mice, and ³H-thymidine incorporation was assayed. Note that 36-5 splenocytes do not respond to syngeneic stimulation. **e**, MLC-stimulated K14 splenocytes have I-A^b-specific cytotoxic activity.

METHODS. For measurement of splenocyte proliferation, freshly isolated splenocytes (4×10^5 unless noted) were cultured in triplicate in 200 μ l RPMI 1640 medium (Cellgro) +10% fetal calf serum (HyClone) with irradiated (2,000 rads) splenocytes as stimulators (**a**, **d**). Alternatively, total spleen or thymus was stained with TIB211 (anti-CD8) followed by depletion with rabbit complement and cells were cultured with 2×10^4 mitomycin C-treated L-cell transfectants (**b**, **c**) in 96-well tissue-culture plates (Costar). Splenocytes were activated for 60 h with 1μ Ci ³H-thymidine (NEN) added to each well for the final 16 h (**a**, **d**). Alternatively (**b**, **c**), 50 μ l of supernatant was removed from each well of the MLC at 48 h and added to 5×10^3 HT-2 cells, an interleukin-2-dependent indicator line. HT-2 cells were cultured for 24 h with 1μ Ci ³H-thymidine added to each well for the final 6 h. To assay cytotoxic activity, spleen cells from unprimed K14/*I-A^{bbb}* (circles), *A_β*^b (+/-) (triangles) or *A_β*^b (-/-) (squares) mice were stimulated in MLCs with irradiated C57Bl/6 stimulator spleen cells. After 5–6 days, the cytotoxic activity was tested by ⁵¹Cr-release of I-A^{bbb} L-cell-transfectants.



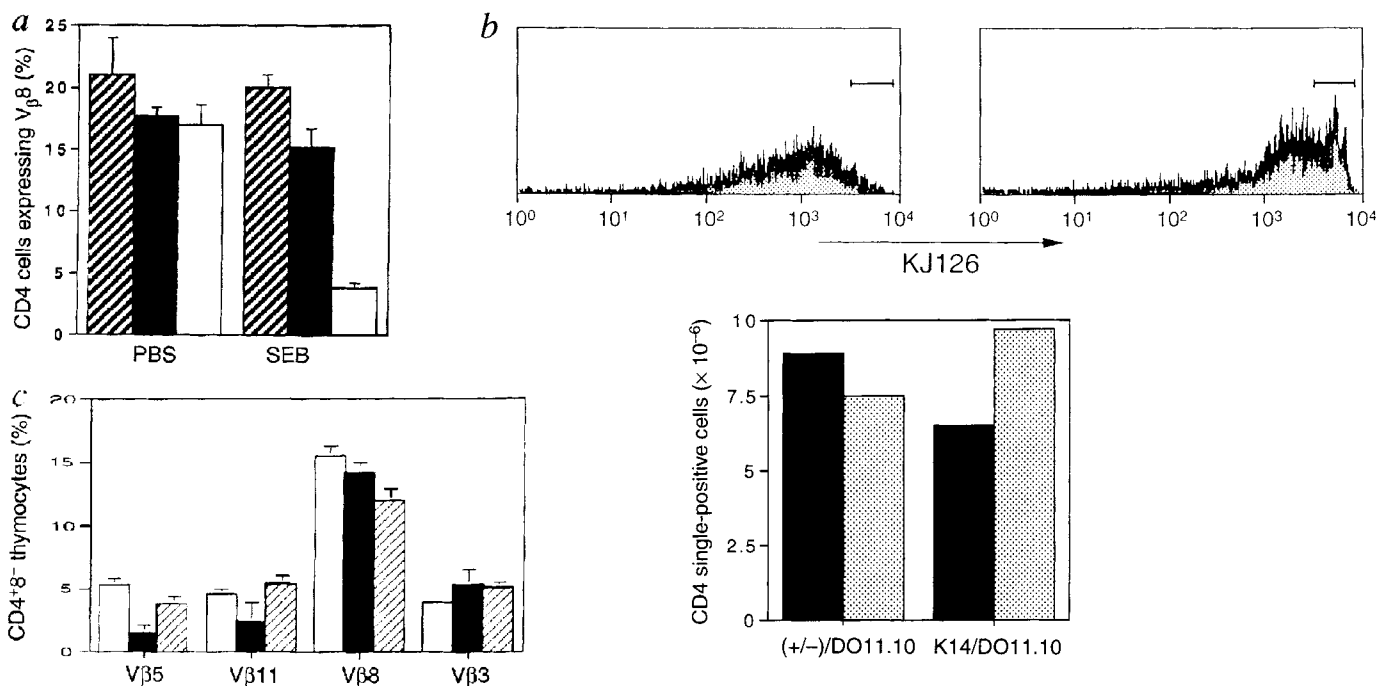


FIG. 3 Clonal deletion does not occur in the absence of thymic medullary selecting elements. **a**, Percentage V β 8⁺ of mature single-positive CD4 T cells in thymi of neonatal A^b (+/-) (white bars), K14/I-A^d (hatched bars), and K14/I-A^b (black bars) mice. **b**, Top, CD4⁺ CD8⁻ DO11.10-positive thymocytes are not deleted by a self-peptide on K14 thymic cortical epithelium. CD4⁺ CD8⁻ cells from A^b (+/-)DO11.10 mice (left) or K14/DO11.10 (right) mice were stained with KJ126, the DO11.10 transgenic TCR clonotype-specific antibody. Note that there are clonotype bright cells present in the K14/DO11.10 thymus but not in the A^b (+/-)DO11.10 thymus. Bottom, CD4⁺ thymocytes are not deleted from DO11.10-transgenic K14 mice on treatment with OVA 323-339 peptide. Thymocytes from A^b (+/-)DO11.10 or K14/DO11.10 mice treated with OVA 323-339 peptide (dotted) or PBS (black) were stained with antibodies to CD4 and CD8, and the numbers of single-positive cells were determined by flow cytometry. The graphs reflect the number of CD4 single-positive cells in each thymus in a representative experiment. **c**, I-E expression restricted to thymic cortical epithelium does not mediate clonal deletion of V β 5- and V β 11-bearing mature thymocytes in $relB$ (-/-) mice. Thymocytes from C57Bl/6 $relB$ (+/-) (white bars), B10.D2 $relB$ (+/-) (black bars) or

B10.D2 $relB$ (-/-) (hatched bars) mice were analysed by flow cytometry. Results are presented as percentage of total CD4 or CD8 single-positive thymocytes. Note that the percentage of V β 5 and V β 11 cells in B10.D2 $relB$ (-/-) thymocytes is not decreased relative to C57Bl/6 $relB$ +/- thymus. **METHODS.** For analysis of SEB-mediated deletion, mice were injected intraperitoneally with 40 μ g staphylococcal enterotoxin B or PBS vehicle alone every other day beginning on day 1 of life. Animals were killed on day 15, and thymocytes were stained with antibodies to CD4 (RM4-5), CD8 (53-6.7) and V β 8.1,8.2 (MR5-2) (Pharmingen). Results are presented as percentage V β 8⁺ of mature single-positive CD4 T cells. For analysis of OVA-peptide-mediated deletion, K14 mice were crossed to DO11.10 TCR transgenic mice. Mice were injected intraperitoneally with 500 μ l of a 100 μ M solution of OVA 323-339 peptide daily for 3 days. After the mice were killed, thymocytes were stained with antibodies to CD4 and CD8 and KJ126, a clonotype-specific antibody to the DO11.10 TCR. The $relB$ (-/-) mice were initially produced on a C57Bl/6 background. They were back-crossed two generations to B10.D2 mice until homozygous for I-A^d, and thymi were analysed by flow cytometry.

It has previously been suggested that superantigen-mediated clonal deletion may be temporally and spatially distinct from peptide-mediated clonal deletion¹⁰. We therefore crossed the K14 mice to DO11.10 TCR transgenic mice to examine peptide-mediated negative selection¹¹. KJ126 clonotype-positive DO11.10 transgenic CD4⁺ T cells proliferated *in vitro* in response to ovalbumin (OVA) peptide 323-339 presented in the context of either I-A^d or I-A^b (ref. 12). Analysis of the double-transgenic mice yielded two insights. First, although K14/DO11.10 thymi contain normal numbers of thymocytes ($1.3 \times 10^8 \pm 1 \times 10^7$), untreated A^b (+/-)DO11.10 thymi have profoundly reduced numbers of thymocytes ($3.5 \times 10^7 \pm 1.3 \times 10^7$). A comparison of the CD4⁺ CD8⁻ thymocytes from control and K14 DO11.10 thymi revealed a population of clonotype-bright double-positive cells only in the latter (Fig. 3b, top). Thus, in the wild-type I-A^b thymus, a population of clonotype-bright double-positive cells is negatively selected in the absence of exogenous OVA peptide. Presumably, the deletion of this population is caused by an unidentified self-peptide. In the K14 mice, thymic cortical epithelium is unable to delete this self-peptide-reactive population. The second insight stems from an assessment of the deletion mediated by exogenous

OVA peptide. Injection of OVA peptide into A^b (+/-)DO11.10 transgenic mice resulted in the further deletion of a population of clonotype-low CD4⁺ cells (Fig. 3b, bottom). In contrast, this OVA peptide-mediated deletion is not observed in the K14/DO11.10 double-transgenic thymus (Fig. 3b, bottom). Indeed, the number of single-positive CD4 cells increased, suggesting additional positive selection mediated by the exogenous peptide. As we noted in the SEB model, peripheral DO11.10⁺ CD4⁺ cells selected in the K14 mouse in the presence of exogenous OVA peptide remained responsive *in vitro* to OVA presented by I-A^b-positive APCs, demonstrating that they are not tolerated (not shown). Thymic cortical epithelium, therefore, cannot mediate either SEB or peptide-induced clonal deletion; in the latter, neither exogenous peptide nor endogenous self-peptide could effect deletion.

We also analysed thymic V β deletion in $relB$ (-/-) mice in which the level of class II expression on thymic cortical epithelium is comparable to that in wild-type mice. As the $relB$ (-/-) mice lack a thymic medulla, they allowed us to isolate and examine the function of thymic cortical epithelium. The $relB$ (-/-) mice were bred to B10.D2 mice, which undergo mtv/I-E-mediated clonal deletion of V β 5 and V β 11 (ref. 13). Flow cytometric analysis of

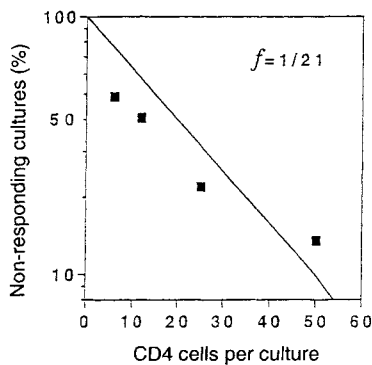


FIG. 4 Precursor frequency analysis of self-reactive CD4 cells from K14 mice. Splenocytes were incubated with anti-CD4-coated dextran beads and CD4⁺ T cells were purified to >90% purity on a MiniMACS column (Miltenyi Biotec). CD4⁺ T cells at varying concentrations were cultured in 200 μ l with 5×10^5 irradiated C57Bl/6 splenic APCs. Cultures were supplemented with 50 U ml⁻¹ rIL-2 (Genzyme) on days 2 and 5, and incubated for 8 days with 1 μ Ci ³H-thymidine added for the last 16 h. Positive cultures were defined as those in which proliferation exceeded the sum of the proliferation of CD4 cells alone and splenic APC alone by 3 standard deviations. The data are plotted in accordance with Poisson statistics by the minimum chi-squared method²⁴.

mature single-positive cells shows that V β 5- and V β 11-positive cells are poorly deleted in B10.D2/*relB* (–/–) thymi (Fig. 3c). The absence of negative selection in the *relB* (–/–) mice demonstrates in a distinct system that thymic cortical epithelium cannot induce negative selection. Similarly, we have previously found a significant reduction in the number of I-E-reactive V β 5- and V β 11-expressing CD4 cells in 36-5 and 2026 E α transgenic mice (which express I-E in the thymic medulla) despite decreased levels of class II expression¹³. These data suggest that the absence of negative selection in the K14 mice is not simply a result of decreased levels of thymic class II expression, as the level of class II expression in the *relB* (–/–) mice is identical to that in wild-type mice, whereas thymic medullary epithelium expressing low levels of class II in the 36-5 and 2026 mice still mediates negative selection.

Previous reports have estimated the rate at which negative selection occurs in the thymic medulla¹⁴. The K14 mice, which undergo positive but not negative selection, allowed us to quantify the extent of negative selection that normally occurs to avoid self-reactivity. The precursor frequency of K14 CD4 cells that proliferate in response to syngeneic splenocytes was determined. Limiting dilution analysis indicated that autoreactive CD4 cells occur at a frequency of 5% (Fig. 4). These data suggest that, at a minimum, one out of every 20 cells that undergoes positive selection must subsequently be negatively selected. These autoreactive cells thus occur at a much higher frequency than do primed antigen-specific, or alloreactive, cells.

The use of K14 mice allows us to quantify the extent of self-reactivity that would occur if positive selection were not followed by negative selection. A much higher frequency of self-reactive cells has been observed in a Y-Ae single-peptide transgenic mouse in which both positive and negative selection occur on only one

peptide determinant¹⁵. Because one peptide dominates the positive selection of CD4⁺ T cells in these mice, the affinity of these cells for self-I-A^b is extremely high. Similarly, the CD4⁺ T cells from DM mutant mice¹⁶, in which most class II molecules are occupied by the class II-associated invariant chain peptide (CLIP) are self-reactive. Positive selection on a broad array of peptides in the K14 mice results in a population of CD4⁺ T cells with a repertoire more closely resembling that of peripheral CD4⁺ T cells in wild-type mice. This more diverse selection environment does not skew the population of selected T cells towards self-MHC molecules, thus providing a more accurate measurement of the development of self-reactive T cells in the normal thymus.

Our data show that there is a distinct phenotypic and functional difference between thymic cortical and medullary epithelium. Previous work has suggested that thymic cortical epithelium is unique in its ability to support the positive selection of CD4 single-positive cells^{17–19}. The absence of both radio-resistant-thymic medullary epithelium and bone-marrow-derived dendritic cells in a *relB* (–/–) mouse suggests that they have a common function⁷. It has been previously demonstrated that expression of an E α transgene by medullary epithelium is not sufficient to achieve positive selection of CD4⁺ T cells^{17,20,21}. In contrast, the analysis of the K14 transgenic mice suggests that thymic cortical epithelium can mediate positive but not negative selection. This is consistent with histological analyses demonstrating that negative selection occurs on haematopoietic cells and medullary epithelium^{14,22,23}. These findings demonstrate that thymocyte development is a sequential process: that positive selection of thymocytes occurs before, independently of, and in distinct anatomical sites from, negative selection. □

Received 7 May; accepted 19 July 1996.

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ACKNOWLEDGEMENTS. We thank C. Reilly for technical assistance; E. Fuchs for the K14 transgene vector; R. Bosch for statistical advice; S. Smiley, M. Kaplan and H. Auchincloss Jr for conversations and reviews of the manuscript; and H. Cantor and J. Sprent for comments on the manuscript. This work was supported by grants from the NIH to D.L., T.M.L. and L.H.G., and a gift from the Mather Foundation to L.H.G.

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The C5a chemoattractant receptor mediates mucosal defence to infection

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A FAMILY of G-protein-coupled chemoattractant receptors is known to mediate the transport and activation of neutrophils and macrophages. This family includes receptors for chemokines, such as interleukin-8, bacterial formylated peptides, platelet-activating factor, leukotriene B₄, and the complement anaphylatoxins¹⁻³. The apparent redundancy of these receptors suggests that they have an important underlying role in host defence. To isolate the contribution of particular molecules, we disrupted a gene that encodes a single chemoattractant receptor. Here we show that mice deficient in the chemoattractant C5a receptor, in comparison to their wild-type littermates, were unable to clear intrapulmonary-instilled *Pseudomonas aeruginosa*, despite a marked increase in neutrophil influx, and succumbed to pneumonia. These C5a-receptor-deficient mice challenged with sublethal inocula of *Pseudomonas* become superinfected with secondary bacterial strains. We conclude that the C5a receptor has a non-redundant function, and is required for mucosal host defence in the lung.

The murine C5a receptor (C5aR) gene⁴ was deleted through homologous recombination with the targeting vector shown in Fig. 1a. Correctly targeted J1 embryonic stem cells were used to generate chimaeric mice, several of which transmitted the targeted allele through the germ line (Fig. 1b). To confirm that C5aR was not present in the targeted animals, binding and signalling to exogenous recombinant mouse C5a and northern blot analyses were performed (Fig. 1c, d). Northern analysis of bone-marrow messenger RNA from rC5aR-deficient (C5aR^{-/-}) mice demonstrated that there was no hybridization with the C5aR complementary DNA coding sequence probe (Fig. 1d).

The C5a receptor is found in all myeloid cell lineages, as well as in hepatocytes and epithelial cells⁵. No developmental or biological defects were observed in these cells, apart from their inability to bind and signal to exogenous C5a (Fig. 1c). C5aR-deficient mice bred normally, and displayed no gross defects when maintained under barrier conditions.

To assess the contribution of the C5aR in a model of inflammatory lung reactions, mice were challenged intratracheally with 3.5×10^6 *P. aeruginosa*, and were studied for lethality for six days. Most (10 of 11) C5aR-deficient mice succumbed in the second day after challenge, whereas none of the seven wild-type mice died (Table 1). To evaluate whether this phenotype represented differential susceptibility to the inflammatory mediators of the microorganism (such as lipopolysaccharide, elastases and formyl

TABLE 1 Assessment of mortality after intratracheal instillation of *Pseudomonas aeruginosa* (P.a.)

Genotype	Lethality after injection of 3.5×10^6 P.a. per mouse (deaths/total)	Lethality after injection of 3.5×10^6 P.a. per mouse (deaths/total)
Wild-type	0/7	
C5aR ^{-/-}	10/11*	0/5**

Paired numbers of wild-type and C5aR-deficient mice were isolated from the central colony and observed for mortality every 24 h after challenge for 6 days. * $P < 0.002$; ** $P < 0.001$; proportions were compared using the two-tailed Fisher's exact test.

peptide chemoattractant factors)^{6,7}, we challenged mice with equal numbers of formalin and heat-killed bacteria. As shown in Table 1, all five knockout (C5aR-deficient) mice survived this treatment.

These data demonstrate that the viability of the microorganism is a significant factor in mortality, and suggest that the C5aR-deficient mice may not be able to clear the microorganism. To examine this possibility, wild-type and knockout mice were given 3.5×10^6 *P. aeruginosa* intratracheally. Some were killed immediately, some after 6 h, and the remainder after 24 h, and their lung homogenates were quantitatively cultured. The results for seven wild-type or C5aR-deficient mice at each time point of the experiment are shown in Table 2. Wild-type mice had begun to clear organisms at 6 h, but the number of *P. aeruginosa* in the C5aR-deficient mice increased. When examined after 24 h, up to 99% of the initial bacterial burden was cleared in wild-type mice, but knockout mice continued to demonstrate bacterial survival and growth. Thus the C5a receptor seems to be essential for clearance of microorganisms.

Polymorphonuclear neutrophils (PMNs) are essential for killing bacteria, and their defects in margination are associated with infection⁸⁻¹⁰. We hypothesized that, in the absence of a chemoattractant receptor, an inadequate inflammatory response might account for the lack of bacterial clearance. The lungs from nine C5aR-deficient or wild-type mice assessed for bacterial survival were washed, the neutrophils counted, and the lungs processed for histological examination. We observed a surprising and paradoxical increase in the number of neutrophils recovered from the C5aR-deficient mice (Fig. 2a). These results were supported by histological analysis, which revealed a dense neutrophil consolidation in the knockout animals that exceeded the response seen in the wild type (Fig. 3a-c). When vascular permeability was assessed by quantification of Evans blue dye, there was found to be an enhanced microvascular leak in the C5aR-deficient mice (Fig. 2b). Despite a brisk neutrophil infiltrate mediated through redundant chemoattractants and enhanced microvascular leak, C5aR-deficient mice do not clear the instilled *P. aeruginosa* organisms, resulting in pulmonary injury and death.

When mice were challenged with a sublethal inoculum of *Pseudomonas* organisms (3.5×10^5) and maintained for six days under non-barrier isolation, further evidence for the importance of the C5aR in mucosal defence was obtained. Quantitative culture of lung homogenates from C5aR-deficient mice at six days post-infection indicated a new pattern of bacterial superinfection with Gram-positive cocci, including two species of *Streptococcus* and a strain of *Staphylococcus* (data not shown), whereas lung homogenates from wild-type mice showed no growth of microorganisms at all. Thus, although the acute infection with *Pseudomonas* was cleared at lower inocula, the deletion of the C5a receptor selectively

TABLE 2 Pulmonary clearance after intratracheal instillation of *P. aeruginosa*

Genotype	CFU ₆ /CFU ₀ 6 h after P.a. injection	CF ₂₄ /CFU ₀ 24 h after P.a. injection installation
C5aR ^{-/-}	$8.2 \pm 3.1 \times 10^6 / 2.8 \pm 1.1 \times 10^6$ *	$2.0 \pm 0.8 \times 10^7 / 3.3 \pm 0.6 \times 10^6$ **
Wild type	$2.3 \pm 1.0 \times 10^6 / 3.0 \pm 0.8 \times 10^6$	$1.5 \pm 0.1 \times 10^5 / 3.1 \pm 0.3 \times 10^6$

Lung clearance of *P. aeruginosa* (P.a.) was measured by killing one group of mice immediately after intratracheal instillation of 3.5×10^6 *P. aeruginosa* (CFU₀) and the other groups either 6 or 24 h later (CFU₆ or CFU₂₄). Lung homogenates were plated and the number of CFU determined. Results are expressed as CFU₆/CFU₀ or CFU₂₄/CFU₀ for independent C5aR-deficient mice and wild-type controls. * $P < 0.01$; ** $P < 0.02$.

predisposed mice to colonization or superinfection with secondary pathogens.

These data uncover an unexpected role for the C5a chemoattractant receptor in mucosal host defence, and demonstrate that the influx of neutrophils is not on its own sufficient to clear the lung of bacteria.

To assess the *in vitro* phagocytic response of neutrophils from receptor-deficient and wild-type mice, a sterile peritoneal inflammation was initiated with glycogen. When C5aR-deficient mice

were tested, a decreased number of neutrophils were elicited: the number of neutrophils that migrated to the peritoneum was one-sixth that of wild-type mice (wild type, $2.94 \times 10^6 \pm 0.76 \times 10^6$ neutrophils, mean $\pm$ s.e.m., $n = 5$; C5aR-deficient, $0.46 \times 10^6 \pm 0.17 \times 10^6$ neutrophils, mean $\pm$ s.e.m., $n = 4$; $P < 0.02$). Thus this 'non-specific' inflammatory stimuli depends partly on intraperitoneal complement activation to extravasate blood neutrophils. However, when such cells were assessed using a phagocytosis assay⁹ for their ability to kill viable *P. aeruginosa in vitro*, no

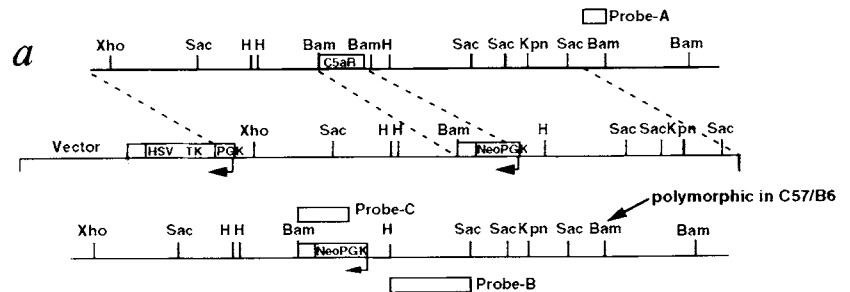
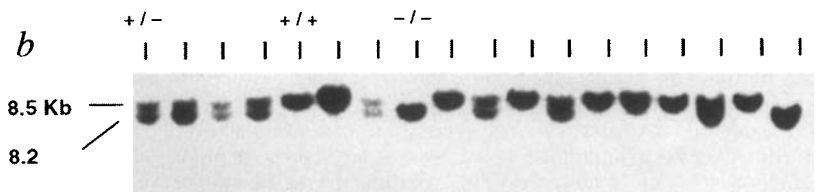
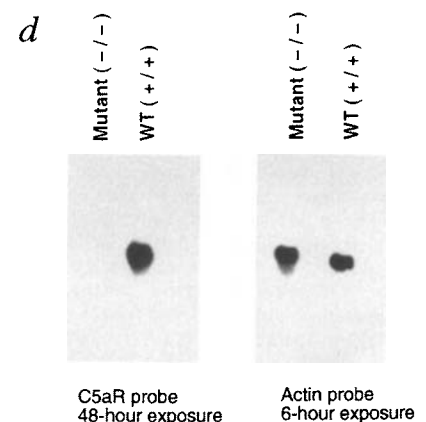
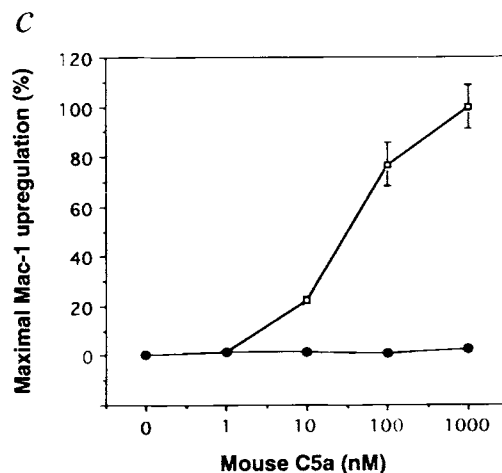


FIG. 1 Targeted disruption of mouse C5a receptor. **a**, Wild-type loci, targeting vector, and mutant loci of the mouse C5aR gene. The wild-type gene is shown containing the coding sequence of the receptor as an open block bounded by two *Bam*HI sites. The entire coding region of the C5a receptor was replaced by a neomycin resistance cassette (pGK-Neo^r) in the targeting vector, replacing the 3' *Bam*HI site. Characteristic *Bam*HI fragments were polymorphic for the C5aR in strain 129 versus C57BL/6. Homologous recombination led to a diagnostic map pattern using a probe outside the targeting vector (probe A), or using the Neo^r probe or target probe within the targeted sequence (probes B and C). **b**, Southern blot analysis of tail DNA from littermates. Genomic DNA was digested with *Bam*HI and hybridized with flanking probe A. **c**, Stimulation of Mac-1 expression on murine neutrophils from wild-type and C5aR-deficient mice in response to recombinant mouse C5a. Expression of Mac-1 is shown using FITC-conjugated rat anti-mouse Mac-1 monoclonal antibodies. Data are expressed as the percentage maximal mean channel fluorescence minus the fluorescence of non-binding isotype-matched control. Open squares represent neutrophils derived from wild-type mice, filled circles represent neutrophils from C5aR-deficient mice. **d**, Northern blot analysis of C5aR expression levels in wild-type (WT +/+) and homozygote (Mutant -/-) animals. Total RNA was prepared from bone-marrow cells of two mice of each genotype, and probed with a ³²P-labelled, 1-kb mouse C5aR cDNA fragment. Blots were stripped and reprobed with a probe specific for β -actin.

	Expected sizes (kb)		
	<i>Bam</i> HI Probe-A	<i>Sac</i> I Probe-B	<i>Sac</i> I Probe-C
Wild type	129	6.5	7.3
Wild type C57/B6	8.5	7.3	No band
Mutant	8.2	7.9	7.9



<i>Bam</i> HI digest with probe A	
WT allele	8.5
Mutant allele	8.2



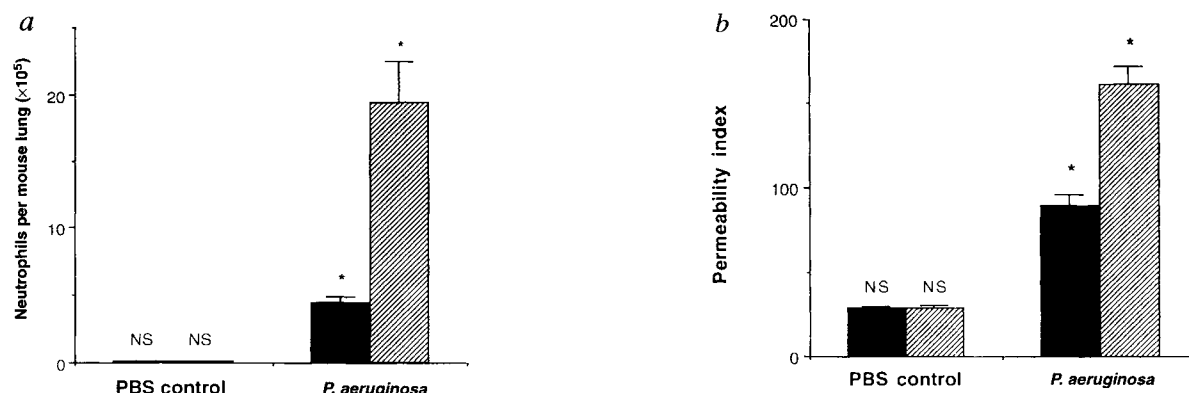


FIG. 2 Microvascular injury induced by intratracheal instillation of *P. aeruginosa*. *a*, Neutrophil accumulation in the lung 6 h after intrapulmonary instillation of *P. aeruginosa* in C5aR-deficient (hatched bars) or wild-type littermate (black bars) mice. Control animals received phosphate-buffered saline (PBS). Asterisk, $P < 0.0002$; NS, not significant. *b*, Lung injury as defined by leakage of protein-bound Evans blue dye 6 h after

intrapulmonary instillation of 3.5×10^6 *P. aeruginosa* in C5aR-deficient (hatched bars) or wild-type (black bars) littermates. Control mice received PBS instead of *P. aeruginosa*. Asterisk, $P < 0.0001$. Values are means $\pm$ s.e.m. for 9 individual C5aR-deficient or wild-type littermate mice; probabilities are on comparison of C5aR-deficient with wild-type mice, as determined by the unpaired Student's *t*-test.

significant differences were observed (data not shown).

We next investigated whether the defect in bacterial clearance was limited to the lung mucosa or represented a more generalized defect. Mice were challenged intraperitoneally with *Pseudomonas* organisms (10^7), and the bacterial clearance and PMN influx were assessed at 0, 6 and 24 h. No differences were observed in the influx of inflammatory cells between wild-type and knockout mice. When clearance was assessed quantitatively, there was a decrease in the clearance of *Pseudomonas* after 6 h in the C5aR-deficient mice (wild-type, $\leq 10^2$ colony-forming units (CFU); C5aR-deficient, $1-1.6 \times 10^4$ CFU; $n = 3$ in each group), but after 24 h essentially the entire inoculum was cleared in both groups. We conclude that in the mucosal surface of the lung the clearance of microorganisms requires the C5a receptor, but in the peritoneum this receptor is accessory or redundant.

Pseudomonas species colonize the lung in cystic fibrosis, and incite a vigorous inflammatory response that results in organ damage^{11,12}. Mice lacking the C5aR recapitulate the phenotype seen in cystic fibrosis patients: ineffective killing of *P. aeruginosa*, despite a vigorous and ultimately toxic neutrophil inflammatory response. Earlier studies with C5-deficient mice, which lack both the ligand C5a as well as the lytic C5b-9 complex, indicated that mice were susceptible to toxicity with the bacteria *Staphylococcus* spp., *Haemophilus influenzae* and *Pseudomonas* spp.¹³⁻¹⁵. These observations represent a phenocopy of the infection seen in cystic fibrosis patients, most of whom harbour the same infective species. As we have shown here, the C5a receptor appears to be important in mediating PMN-dependent clearance of the mucosal

bacterial organisms. We propose that neutralization of the interaction between C5a and the C5a receptor in cystic fibrosis may be an important factor in allowing successful colonization of the lung with *P. aeruginosa* species. □

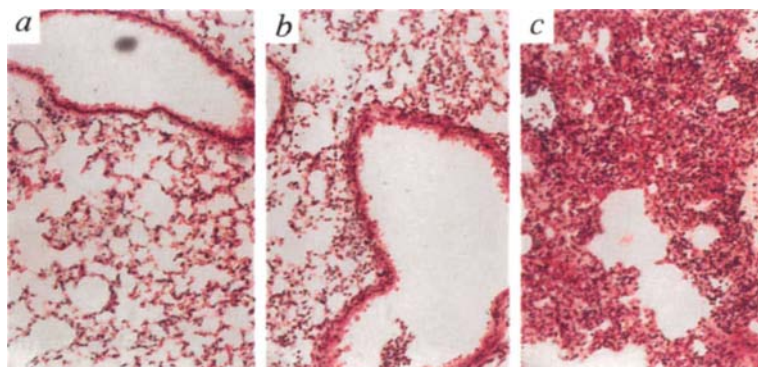
Methods

Animal studies. These were performed according to institutional and NIH guidelines for animal use and care.

Targeted disruption of mouse C5a receptor. The targeting vector was constructed by subcloning the C5a receptor gene⁴ into pBluescript as a 13.2-kilobase (kb) insert isolated from a 129 strain genomic library. A construct was derived from this plasmid which deleted a 1.2-kb *Bam*HI fragment containing the entire coding region of the C5a receptor. The deleted section was replaced with a pGK-Neo resistance cassette. The targeting vector contained 5.9 kb of 5' sequence and 6.1 kb of 3' sequence flanking the *neo* cassette. The targeting fragment was subcloned into the pPNT vector for positive/negative selection with geneticin and gancyclovir. Homologous recombination was screened by treating cells with restriction enzymes *in situ*. Analysis by both single (G418) and double selection was performed yielding 17 targeted clonal lines from 100 doubly resistant colonies picked. Five of these lines were analysed for karyotype, and two were selected for blastocyst injection into C57BL/6 mice. Chimaeric males were bred to yield germline transmission of the targeted allele.

Northern blot analysis. Total RNA was prepared from peritoneal cells and bone-marrow cells of two mice of each genotype, matched in age and gender. The RNA (10 μ g) was separated on formaldehyde agarose gels, transferred to GeneScreen membranes (DuPont) and probed with a ³²P-labelled 1-kb mouse C5aR cDNA fragment. Filters were additionally probed with a specific probe for β -actin.

FIG. 3 Histology of C5aR-deficient and wild-type mice after challenge with *P. aeruginosa*. *a*, The lungs of wild-type and C5aR-deficient control mice seemed normal 6 h after challenge with PBS (wild type depicted, haematoxylin-eosin stain; original magnification, $\times 200$). *b*, At 6 h after intratracheal instillation of 3.5×10^6 *P. aeruginosa*, wild-type mice showed mild inflammatory infiltrates with neutrophils in the alveolar spaces and the airways (haematoxylin-eosin stain; original magnification, $\times 200$). *c*, The lung of a C5aR-deficient mouse 6 h after intratracheal instillation of 3.5×10^6 *P. aeruginosa* showed widespread inflammation with neutrophils, haemorrhage, and fluid-filled alveoli evident (haematoxylin-eosin stain; original magnification, $\times 200$).



Mac-1 upregulation on neutrophils. Stimulation of Mac-1 expression on murine neutrophils was performed as described¹⁶. In brief, whole blood anticoagulated with sodium citrate was obtained by intracardiac puncture from killed mice of each genotype. Blood (100 µl) was incubated with various concentrations of recombinant mouse C5a-Flag for 10 min at 37 °C. The cells were then pelleted at 200g and incubated with either fluorescein isothiocyanate-conjugated rat anti-mouse Mac-1 monoclonal antibody or rat IgG-2b isotype-matched control antibody for the mouse cells at 4 °C for 30 min. The red blood cells were lysed and the remaining cells washed. The cells were then counted immediately in a flow cytometer with a live gate on neutrophils. Data from 5,000 cells per sample were recorded and expressed as the percentage maximal mean channel fluorescence minus the fluorescence of the non-binding isotype control antibody.

Bacteria. The mucoid *P. aeruginosa* strain AO1 was used for all studies. Stock culture portions of a log-phase culture were maintained in skimmed milk at -70 °C. *Pseudomonas* was grown in LB medium for 18 h before the study, and washed twice in sterile saline. Bacterial suspensions were produced with the desired concentration of bacteria determined by optical density from previous growth curves. The concentration of bacteria was confirmed by streaking serial dilutions of bacterial suspensions on LB agar or occasionally on Centrimide-agar, a specific growth agar for *P. aeruginosa* that does not allow growth of other bacterial strains.

Animal model of *P. aeruginosa* lung infection. Before challenge with *P. aeruginosa*, the mice (matched for gender and age) (8 weeks) were anaesthetized with ketamine (90 mg per kg body weight) and xylazine (10 mg per kg). *P. aeruginosa* (3.5×10^6) was instilled intratracheally in a volume of 50 µl. Then, 2 h after the *P. aeruginosa* installation, 100 µl of Evans blue (6.25 mg ml⁻¹) was administered in the tail vein to assess permeability changes. At varying time points after challenge, mice were killed. Bronchopulmonary lavage was performed 6 or 24 h after challenge with *P. aeruginosa*, using the same procedure as described above. The recovered bronchoalveolar lavage fluid was assessed for differential cell counts and cytokine measurement. Permeability changes were determined by comparing leakage of Evans blue into lung lavage fluid to the amount remaining in 1 ml plasma¹⁷. Lung sections were prepared for histological analyses after inflation of the lung with 10% neutral buffered formalin.

Assessment of mortality. Paired numbers of wild-type and C5aR-deficient mice were isolated from the central colony and observed for mortality every 24 h after challenge for 6 days. The mice were given either 3.5×10^6 live or 3.5×10^6 formalin-treated and heat-shocked *P. aeruginosa*.

Pulmonary clearance of *P. aeruginosa*. The lung clearance of *P. aeruginosa* was measured by killing one group of mice immediately after intratracheal instillation of 3.5×10^6 *P. aeruginosa* and the other groups either 6 or 24 h later. The lungs were excised and homogenized in 4 ml of ice-cold distilled water. Homogenates were diluted appropriately in sterile saline and cultured on LB plates, and the number of CFU determined. Results were expressed as CFU_g/CFU₀ or CFU₂₄/CFU₀.

Received 20 May; accepted 16 July 1996.

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ACKNOWLEDGEMENTS. We thank L. Kobzik, G. Pier and P. Barrett for *Pseudomonas* expertise and help with histological analyses; B. Rollins, T. Schall, P. Weller and H. Showell for discussions; B. Kolier for blastocyst injections; and C. Bozic and A. Rehm for reading the manuscript. This work was supported in part by grants from the NIH and the Rubenstein-Cable Cystic Fibrosis Research Fund at the Perimutter Laboratory. U.E.H. was supported by a fellowship from the Deutsche Forschungsgemeinschaft.

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Control of calcium oscillations by phosphorylation of metabotropic glutamate receptors

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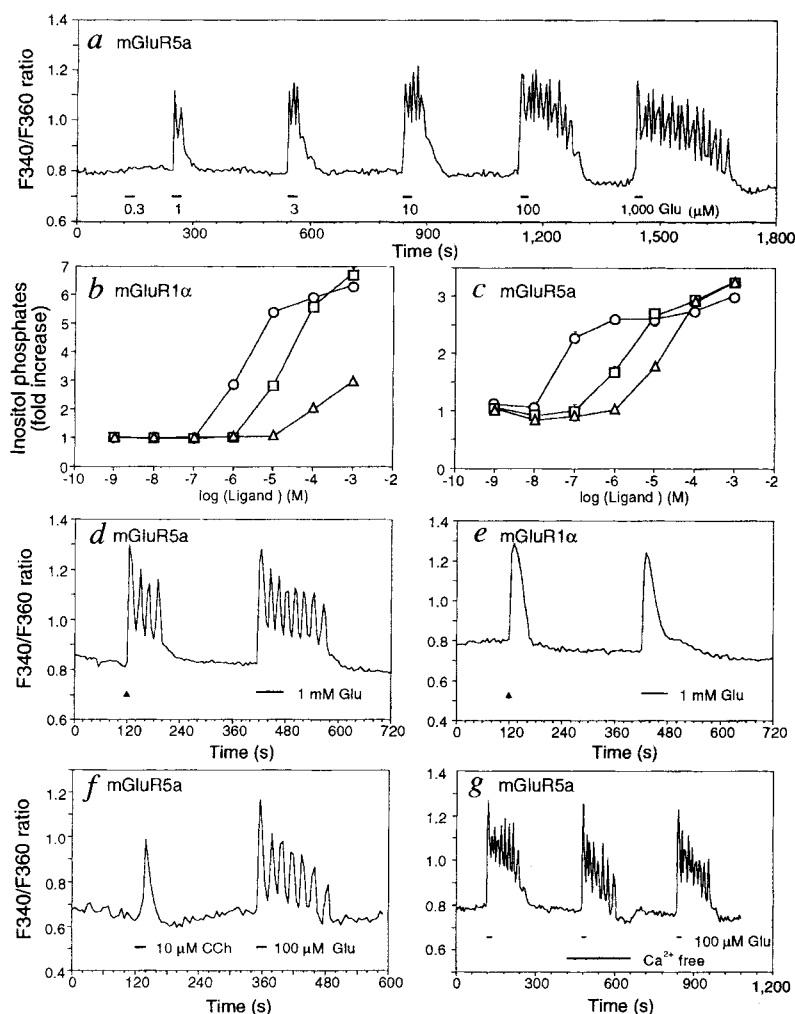
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STIMULATION of two metabotropic glutamate-receptor subtypes, mGluR1 and mGluR5, triggers the release of Ca²⁺ from intracellular stores through the inositol-(1,4,5)trisphosphate (InsP₃) pathway^{1–3}. Here we report that glutamate induces single-peaked intracellular Ca²⁺ mobilization in mGluR1α-transfected cells but elicits Ca²⁺ oscillations in mGluR5α-transfected cells. The response patterns of the intracellular Ca²⁺ increase depend upon the identity of a single amino acid, aspartate (at position 854) or threonine (at position 840), located within the G-protein-interacting domains of mGluR1α and mGluR5α, respectively. Pharmacological and peptide mapping analyses indicated that phosphorylation of the threonine residue at position 840 of mGluR5α by protein kinase C (PKC) is responsible for the generation of Ca²⁺ oscillations in mGluR5α-expressing cells. To our knowledge this is the first evidence that PKC phosphorylation of G-protein-coupled receptors is important in producing oscillations in intracellular Ca²⁺ signalling.

Two cell lines, HEK293 and NIH3T3 cells, were stably transfected with complementary DNA encoding rat mGluR1α or mGluR5α, and increases in [Ca²⁺]_i elicited by glutamate exposure were analysed by single-cell Ca²⁺ recording. In both cell lines, glutamate induced a single-peaked, non-oscillatory [Ca²⁺]_i increase in mGluR1α-expressing cells. The number of oscillatory peaks in mGluR5α-expressing cells increased in a concentration-dependent manner (Fig. 1a). This oscillatory response was never observed at the same range of glutamate concentrations in mGluR1α-expressing cells (for example, see Fig. 1e). The rank order of the effectiveness of various agonists to induce phosphatidylinositol hydrolysis was identical for mGluR1α and mGluR5α, and the affinities for the agonists were slightly higher for mGluR5α than for mGluR1α (Fig. 1b, c). Oscillatory and non-oscillatory patterns of [Ca²⁺]_i increase, however, were independent of the duration of glutamate application (Fig. 1d, e), and carbachol (1–100 µM) evoked a single-peaked [Ca²⁺]_i increase through endogenous muscarinic receptors in HEK293 cells expressing the oscillatory type of mGluR5α (Fig. 1f). Thus, oscillatory and non-oscillatory patterns of [Ca²⁺]_i increase depend on receptor identity. [Ca²⁺]_i oscillations were observed in Ca²⁺-free medium (Fig. 1g) and treatment with 10 nM thapsigargin, a drug that depletes [Ca²⁺]_i from intracellular stores⁴, completely abolished glutamate-induced [Ca²⁺]_i increases in mGluR5α-expressing cells (*n* = 3; not shown). The oscillatory [Ca²⁺]_i response thus derives solely from the mobilization of Ca²⁺ from intracellular stores.

Characterization of a series of chimaeric receptors between mGluR1α and mGluR5α identified a sequence of 19 amino acids (i4) which is located just distal to transmembrane-seven and which determined the response pattern of the chimaeric receptor (Fig. 2a, c, d). This region is important for receptor–G-protein interaction⁵ and differs by only one amino acid in mGluR1α (aspartate at position 854) and mGluR5α (threonine at position 840) (Fig.

FIG. 1 Fluorometric measurements of $[Ca^{2+}]_i$ increases elicited by glutamate and dose-response curves of agonist-stimulated phosphatidylinositol hydrolysis. **a**, HEK293 cells expressing mGluR5a were exposed to various concentrations of glutamate for 20 s. **b**, **c**, Dose-response curves of agonist-stimulated phosphatidylinositol hydrolysis were determined in NIH3T3 cells expressing mGluR1 α (**b**) and mGluR5a (**c**) by measuring the accumulation of total inositol phosphate formation after incubation of quisqualate ($\circ$), glutamate ($\square$) and (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylate ($\triangle$) for 20 min (ref. 3). The values are means of triplicate determinations. **d**, **e**, $[Ca^{2+}]_i$ responses were recorded by exposing 1 mM glutamate to a clonal HEK293 cell expressing mGluR5a (**d**) or mGluR1 α (**e**) for 1 s (arrowheads) and 60 s (bars). **f**, A clonal HEK293 cell line expressing mGluR5a was exposed to either 10 μ M carbachol (CCh) or 100 μ M glutamate (Glu) for 20 s. **g**, $[Ca^{2+}]_i$ responses triggered by 100 μ M glutamate for 20 s were recorded in a clonal HEK293 cell expressing mGluR5a in a Ca^{2+} -containing solution or in a Ca^{2+} -free solution containing 0.1 mM EGTA. **METHODS**. cDNA encoding either rat mGluR1 α ¹ or mGluR5a² was inserted into the eukaryotic expression vector pEF-BOS²⁴. HEK293 cells and NIH3T3 cells expressing mGluR1 α and mGluR5a were selected with 400 μ g per ml geneticin after transfection of the above plasmids and isolated by single cloning. These cells were loaded for 45 min with Fura-2-acetoxymethyl ester²⁵ (6 μ M) dissolved in a solution (BSS) containing 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$ and 10 mM HEPES buffer (pH 7.4). After incubation, the cells were placed on an inverted-stage microscope and continuously perfused with BSS (32 °C; 2 ml min⁻¹). $[Ca^{2+}]_i$ measurements were made as described²⁶. The fluorescence ratio obtained at 340 and 360 nm (F340/F360) was used as an index of $[Ca^{2+}]_i$.



2b). The presence of threonine generates a consensus PKC recognition site (TVVR in single-letter amino-acid code)⁶, suggesting a role for PKC in the oscillatory response pattern of mGluR5a.

The possibility of PKC involvement in the $[Ca^{2+}]_i$ oscillatory response was investigated pharmacologically. A protein kinase inhibitor, H-7 (100 μ M) (ref. 7), nearly eliminated the $[Ca^{2+}]_i$ oscillations in mGluR5a-expressing cells (Fig. 3a), whereas HAI1004 (100 μ M) (a weaker inhibitor of PKC than H-7, but with similar potency against protein kinase A and protein kinase G⁷) had no effect (Fig. 3c). The more potent, PKC-preferring inhibitor staurosporine⁸ (100 nM) completely abolished oscillatory responses of both mGluR5a and mGluR1 α -(i4) (Fig. 3d, e). None of these compounds showed any effect on $[Ca^{2+}]_i$ increase due to mGluR1 α (Fig. 3b, f). A specific calmodulin-dependent protein kinase II inhibitor KN-62 (1 μ M) (ref. 9) had no effect on the $[Ca^{2+}]_i$ oscillatory pattern of mGluR5a (Fig. 3g). A PKC activator, phorbol-12-myristate-13-acetate (PMA) (ref. 10), abolished $[Ca^{2+}]_i$ responses in mGluR5a- and mGluR1 α -(i4)-expressing cells (Fig. 3h), whereas it reduced but never abolished $[Ca^{2+}]_i$ increases in mGluR1 α - and mGluR5a/1 α -(i4)-expressing cells (Fig. 3h). An ineffective PKC analogue, 4 α -phorbol-12-myristate-13-acetate¹¹, had no effect on either mGluR1 α or mGluR5a, at least up to 300 nM ($n = 6$; not shown). These studies clearly indicate the importance of PKC in the mechanism of oscillatory response.

To investigate the possibility of phosphorylation by PKC within the i4 sequence of each subtype, two peptides covering the i4 sequences of mGluR1 α (residues 832–847) and of mGluR5a (residues 846–861) were chemically synthesized and phosphorylated by PKC *in vitro*. The mGluR5a peptide was phosphorylated

about fourfold more than the mGluR1 α peptide (Fig. 4a), and the K_m value for the mGluR5a peptide phosphorylation ($58.2 \pm 22.1 \mu$ M, $n = 3$) was within the K_m values reported for other PKC phosphorylation substrates¹². Differential PKC phosphorylation of the two subtypes was also investigated in receptor-expressing cells. The mGluR5a and mGluR5a/1 α -(i4) receptor proteins (relative molecular masses, 145K) were isolated by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) after immunoprecipitation (Fig. 4b) and their tryptic digests subjected to two-dimensional phosphopeptide mapping (Fig. 4c, d). The two receptor proteins showed three common phosphopeptides, but mGluR5a gave rise to an additional tryptic fragment (spot 1) which precisely co-migrated with a synthesized phosphopeptide of amino-acid residues 834–843 of mGluR5a, determined by prediction of trypsin digestion sites (Fig. 2b). The result thus indicates that the PKC site at threonine 840 is recognized by PKC in cells.

$[Ca^{2+}]_i$ oscillations through the $InsP_3$ – Ca^{2+} cascade of various G-protein-coupled receptors have been shown to result from the positive and negative feedback effects of $[Ca^{2+}]_i$ mobilization on $InsP_3$ receptors^{13–16}. The effects of PKC activators and inhibitors on $[Ca^{2+}]_i$ oscillations have also been reported^{17–21}, but no target proteins responsible for PKC-mediated $[Ca^{2+}]_i$ oscillations have yet been identified. This study demonstrates a new mechanism in which a single amino-acid substitution in the G-protein-interacting domains of two mGluR subtypes determines the susceptibility to PKC phosphorylation and the ability to transduce either an oscillatory or non-oscillatory Ca^{2+} signal. We propose as a possible mechanism of mGluR5-mediated $[Ca^{2+}]_i$ oscillations that phosphorylation at threonine (position 840) by PKC prevents

further InsP_3 formation by interfering with signal transduction between the receptor and G protein, and that subsequent dephosphorylation regenerates an increase in InsP_3 formation by restoring the signal transduction. For mGluR1, our model predicts that non-phosphorylation at aspartate (position 854) should produce a

non-oscillatory and PKC activator-resistant $[\text{Ca}^{2+}]_i$ response. Whether or not phosphorylation and dephosphorylation of mGluR5 can occur rapidly enough to generate $[\text{Ca}^{2+}]_i$ oscillations is unknown. It has, however, been reported that in cultured astrocytes in which mGluR5, but not mGluR1, is expressed in

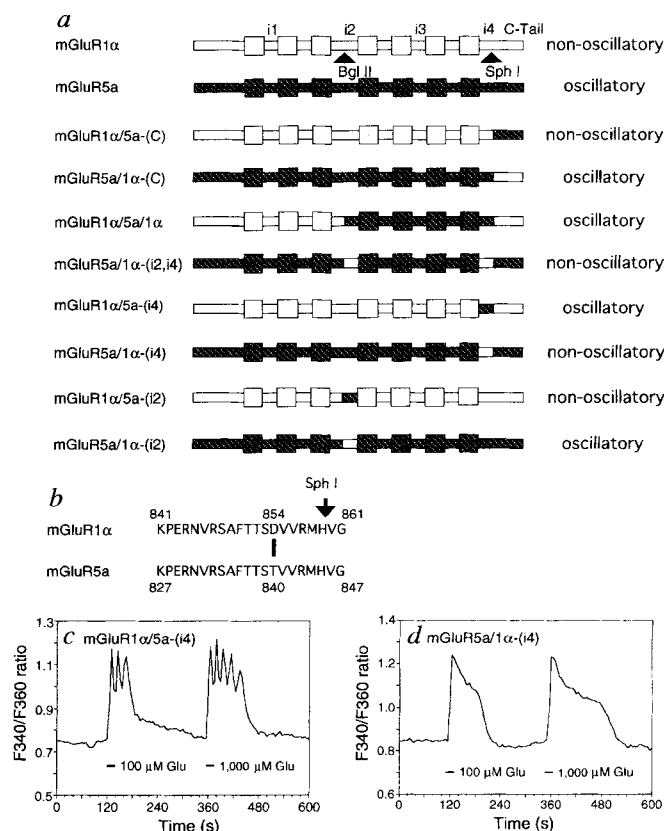
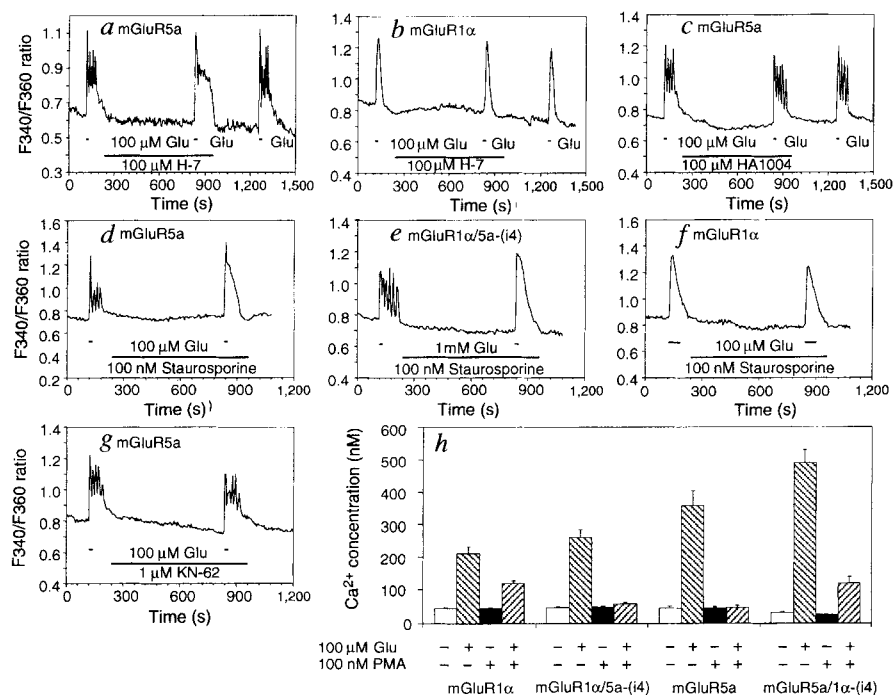


FIG. 2 Patterns of increases in $[\text{Ca}^{2+}]_i$ elicited by glutamate in cells transfected with chimaeric receptors. **a**, The structures of the chimaeric receptors used are shown on the left and their patterns of increase in $[\text{Ca}^{2+}]_i$ in receptor-expressing HEK293 cells and NIH3T3 cells are indicated on the right. Shaded and non-shaded regions represent the sequences derived from mGluR5a and mGluR1α, respectively; i1, i2, i3, i4 are the first, second and third intracellular loops, and the 19-amino-acid sequence of the C-terminal intracellular tails, respectively. Note that replacements of the sequences containing i4 between mGluR1α and mGluR5a change the response patterns of the $[\text{Ca}^{2+}]_i$ increase. **b**, The residue connected by a bar shows a single amino-acid substitution between the i4 sequences of mGluR1α and mGluR5a. **c**, **d**, Patterns of $[\text{Ca}^{2+}]_i$ increase in HEK293 cells expressing mGluR1α/5a-(i4) and mGluR5a/1α-(i4).

METHODS. For construction of the chimaeric receptors, mGluR1α/5a-(i2) and mGluR1α/5a-(i4), the i2 or i4 regions of the mGluR5a receptor were incorporated into the mGluR1α sequence using the polymerase chain reaction (PCR). A BglII site was introduced using the PCR into the mGluR5a sequence at Lys 687 and the BglII-SphI fragment from this mutant was substituted for the equivalent fragment in mGluR1α to make mGluR1α/5a/1α. The mGluR5a with BglII site was used to subclone BglII-SphI fragments in which either i2, i4 or both from the mGluR1α was incorporated into the mGluR5a sequence by PCR (to yield mGluR5a/1α-(i2), mGluR5a/1α-(i4), and mGluR5a/1α-(i2, i4)). Two complementary chimaeras (mGluR1α/5a-(C) and mGluR5a/1α-(C)) were generated by exchanging the C-terminal tails of these two receptors, using the SphI site.

FIG. 3 Effects of protein kinase inhibitors and activator on patterns of $[\text{Ca}^{2+}]_i$ increases. The effects of various protein kinase inhibitors on $[\text{Ca}^{2+}]_i$ increases in HEK293 cells expressing mGluR5a (**a**, **c**, **d**, **g**), mGluR1α (**b**, **f**) and mGluR1α/5a-(i4) (**e**). In these experiments, cells were exposed to 100 μM glutamate (Glu) for 20 s (**a**–**d**, **g**) or 60 s (**f**), or 1 mM glutamate for 20 s (**e**). **h**, The effects of PMA on glutamate-stimulated $[\text{Ca}^{2+}]_i$ increases were determined in NIH3T3 cells expressing mGluR1α, mGluR1α/5a-(i4), mGluR5a or mGluR5a/1α-(i4). **METHODS.** $[\text{Ca}^{2+}]_i$ measurements were made as for Fig. 1, except for **h**. H-7 and HA1004 were dissolved in H_2O at 10 mM before dilution with BSS. Staurosporine and KN-62 were dissolved in dimethylsulphoxide before dilution with BSS. The final concentration of dimethylsulphoxide (0.1%) had no effects on the pattern of $[\text{Ca}^{2+}]_i$ increase ($n = 2$). In **h**, the fluorescence at 510 nm after excitation at 340 nm or 380 nm was recorded with a fluorescence spectrometer (CAF 100; Japan Spectroscopic), and $[\text{Ca}^{2+}]_i$ was calculated from the F340/F380 ratio as described²⁵; cells were treated with 100 nM PMA for 5 min before the application of 100 μM glutamate.



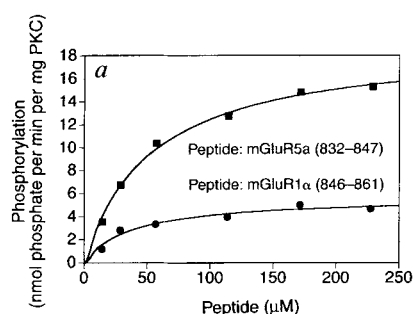
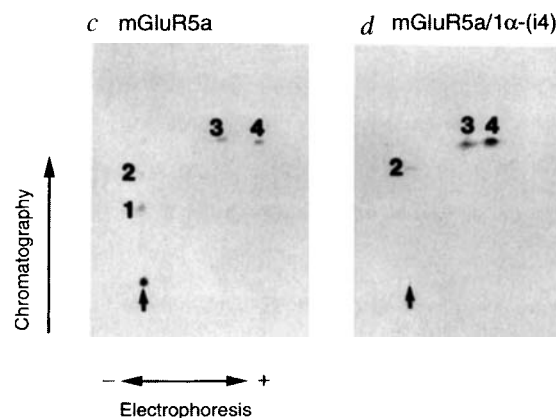
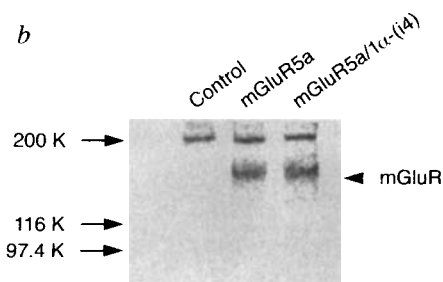


FIG. 4 PKC-dependent phosphorylation of threonine at position 840 of mGluR5a. **a**, PKC-dependent phosphorylation of synthetic peptides corresponding to amino-acid residues 832–847 of mGluR5a and to amino-acid residues 846–861 of mGluR1 α are plotted against the peptide concentrations tested. **b**, SDS-PAGE analysis of immunoprecipitates of solubilized membrane fractions derived from non-transfected (control), mGluR5a-expressing and mGluR5a/1 α -(i4)-expressing cells after treatment with PMA. **c**, **d**, Two-dimensional tryptic phosphopeptide mappings of mGluR5a (**c**) and mGluR5a/1 α -(i4) (**d**). This result is representative of four experiments, and a synthesized phosphopeptide representing amino-acid residues 834–843 of mGluR5a migrated to the position of spot 1. The arrows indicate the origin.

METHODS. For *in vitro* phosphorylation, the above two peptides were synthesized using a PSSM-8 peptide synthesizer (Shimadzu, Japan) and purified by reversed-phase HPLC. The phosphorylation reaction was carried out with 2.5 milliunits of purified rat PKC (Sigma) at 30 °C for 4 min as described¹². For *in vivo* phosphorylation, non-transfected NIH3T3 cells or NIH3T3 cells expressing mGluR5a or mGluR5a/1 α -(i4) were prelabelled with 100 μ Ci/ml [³²P]orthophosphate and treated with 100 nM PMA for 5 min. The mGluRs were solubilized²⁷ from crude membrane preparations and immunoprecipitated by antibody raised against the C-terminal sequence of rat mGluR5a²⁸. The resultant immunoprecipitates were subjected to SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane. The membrane piece containing the 145K mGluR proteins was



excised and incubated for trypsin digestion. Tryptic digests of the purified receptors or the synthetic phosphopeptide of the tryptic sequence of mGluR5a (residues 834–843) were subjected to two-dimensional thin-layer peptide mapping, with an electrophoresis pH of 1.9 at 1,000 V for 25 min, followed by ascending chromatography²⁹.

preference²², glutamate mobilizes intracellular calcium through quisqualate-selective receptors and induces $[Ca^{2+}]_i$ oscillations²³. Thus, $[Ca^{2+}]_i$ oscillations evoked by mGluR5 could contribute to diverging intracellular signalling in glutamate transmission. □

Received 12 April; accepted 21 July 1996.

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ACKNOWLEDGEMENTS. We thank H. Kasai for helpful discussion.

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Spreading of transcriptional repressor SIR3 from telomeric heterochromatin

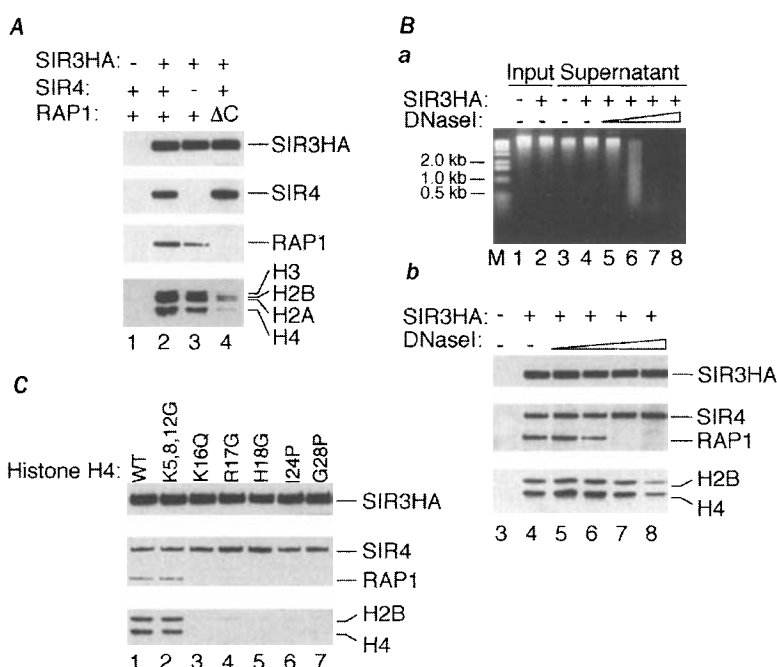
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TELOMERIC genes and the HM loci in *Saccharomyces cerevisiae* are transcriptionally repressed and adopt a heterochromatin-like structure^{1,2}. The *trans*-acting factors RAP1, SIR3 and SIR4 are required for telomeric and HM silencing^{3–5}, and are thought to be chromosomal^{6–8}, but how they contribute to histone-dependent repression of adjacent chromatin^{9–11} is unclear. SIR3 suppresses silencing defects in histones¹⁰, is limiting for silencing adjacent to telomeres¹², and interacts with the H3 and H4 amino termini *in vitro*¹³. Here we show that SIR3 co-immunoprecipitates SIR4, RAP1 and histones from cellular extracts, suggesting the presence of large chromatin-associated protein complexes. Crosslinking experiments show that SIR3 is present at *HMRa*, *HMLa* and telomeres *in vivo*, and that it spreads from telomeric regions into adjacent chromatin when overexpressed. Thus SIR3 is a structural component of yeast heterochromatin, repressing adjacent genes as it spreads along the chromosome.

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FIG. 1 Co-immunoprecipitation of SIR3, SIR4, RAP1 and the core histones. **A**, Whole-cell extracts (WCEs) from strains expressing wild-type (WT) or epitope-tagged SIR3 (SIR3HA) and either deficient for SIR4 or carrying the RAP1-21 mutation (ΔC)¹⁴ were immunoprecipitated with antibodies against SIR3HA²². Precipitates were analysed by western blotting with antisera against the proteins indicated. Two exposures of the same blot probed first for H2B, H3, H4 and then for H2A were superimposed for the histone pattern shown. **B**, DNaseI sensitivity of the SIR3/RAP1/histone interactions. **a**, Ethidium bromide-stained DNA samples from WCEs before (input, lanes 1 and 2) and after immunoprecipitation (supernatant, lanes 3–8) and with (lanes 5–8) or without DNaseI treatment (lanes 1–4); *M*, size standard. **b**, Western blot analyses of immunoprecipitates treated with increasing amounts of DNaseI. **C**, Mutations in the H4 silencing domain affect the SIR3/RAP1/histone interactions. As the anti-H4 antiserum recognizes H4 R17G, H18G and I24P only weakly, coprecipitation of chromatin was monitored additionally with anti-H2B antibodies. **METHODS**. Two copies of the haemagglutinin epitope²² were attached C-terminally to SIR3 by recombinant PCR²³. Multicopy vectors with WT SIR3 or SIR3HA were transformed into AYH2.8 (*Mata*, *ade2*, *his3*, *leu2*, *lys2*, *trp1*, *ura3*, *adh4::URA3 TEL*, *sir3::LEU2*) or LJY155 (ref. 9) (*MATA*, *ade2*, *his3*, *leu2*, *lys2*, *trp1*, *ura3*, *sir3::LEU2*, *hfh1::HIS3*) and isogenic derivatives with mutations in SIR4, RAP1 or H4. WCEs prepared in 50 mM HEPES/KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 and protease inhibitors²⁴ were immunoprecipitated with monoclonal antibodies 17D09 (ref. 22) covalently coupled to Protein A sepharose^{24–26}. Precipitates were separated on 7.5% and 17% SDS-polyacrylamide gels, transferred onto nitrocellulose and sequentially probed (Amersham ECL system) with rabbit antisera generated against H2A, H2B, or glutathione S-transferase



fusions containing residues 1–46 of H3, 1–34 of H4, 1–178 of SIR4, 945–978 of SIR3, or 19–498 of RAP1. DNaseI digests were done during immunoprecipitation with 100, 150, 200 and 250 U DNaseI.

To test the hypothesis that SIR3 spreads from a telomeric heterochromatin-like complex into adjacent chromatin^{12,13}, we investigated whether SIR3 interacts with SIR4, RAP1 and chromatin in cellular extracts. Epitope-tagged SIR3 (SIR3HA) was expressed from high-copy-number plasmids in yeast, and immunoprecipitated from whole-cell extracts. Western blot analyses show that SIR4, RAP1 and histones H2A, H2B, H3 and H4 all co-immunoprecipitate with SIR3HA (Fig. 1A, lane 2). The interactions within this complex show a differential requirement for the presence of DNA. When the complex is extensively digested with DNaseI (Fig. 1Ba, lanes 7 and 8), interactions between SIR3 and SIR4 do not seem to be affected, interactions between SIR3 and histones are moderately affected, and interactions between SIR3 and RAP1 are disrupted completely (Fig. 1Bb, lanes 7 and 8). Because DNA is an integral component of chromatin, and indirect interactions between RAP1 and SIR3 are unlikely (see below), this result implies that DNA helps to maintain the integrity of the RAP1/SIR3/histone complex. All four core histones are present equally after DNaseI digestion (Fig. 1Bb and data not shown), and H2B follows H4 in immunoprecipitations from strains with different H4 mutations (Fig. 1C); this is also true for H2A (not shown). The presence of the core histones and their similar behaviour indicates the precipitation of chromatin fragments rather than individual histones by SIR3HA, and suggests that SIR3 is found in chromosomal complexes.

To characterize further the observed interactions, and to determine the interdependence of the various factors involved, we investigated how mutations in SIR4, RAP1 and H4 affect their coprecipitation with SIR3. The absence of SIR4 reduces the amount of RAP1 and histones coprecipitating with SIR3HA, but does not abolish these interactions (Fig. 1A, lane 3). SIR4 itself can interact with RAP1 and histones^{6,8,13}, which may explain why a fraction of these proteins is lost along with SIR4. The RAP1-21 mutant lacking carboxy-terminal residues 800–827 is defective for silencing, although it can still bind to DNA¹⁴. RAP1-21 no longer coprecipitates with SIR3HA (Fig. 1A, lane 4), thus ruling out an indirect, DNA-mediated interaction between RAP1

and SIR3, and supporting an interaction between the C terminus of RAP1 and SIR3 (ref. 14). Moreover, because the amount of the histones in the immunoprecipitate is partly reduced when RAP1 is defective (Fig. 1A, lane 4), the ability of SIR3 to interact with chromatin is coupled in part to the interaction between RAP1 and SIR3.

Mutations in the charged H4 silencing domain R1 (residues 16–20) and the relatively uncharged domain R2 (residues 21–29) perturb silencing *in vivo*^{5,9–11}. We wished to determine how these mutations influence the interactions between SIR3 and histones. The single amino-acid exchanges K16Q, R17G, H18G, I24P and G28P disrupt the interactions between SIR3 and histones (Fig. 1C, lanes 3–7), whereas the triple mutation K5, 8, 12G, outside R1 and R2 obvious and without silencing phenotype, has no effect (Fig. 1C, lane 2). Therefore only mutations that disrupt silencing also disrupt binding of SIR3 to chromatin. Histone H4 is also involved in the association of SIR3 and RAP1, as RAP1 is lost from the complexes together with mutant H4 (Fig. 1C, lanes 3–7). Along with the effect of the RAP1-21 mutation, this suggests that H4 and RAP1 are both required for the interaction of SIR3 with chromatin. In contrast, coprecipitation of SIR3 and SIR4 is not affected by mutations in either RAP1 or H4 (Fig. 1A, C).

To test whether SIR3 is bound to chromatin *in vivo*, and to map the chromosomal regions with which SIR3 might interact, we immunoprecipitated SIR3HA (expressed from multicopy vectors) from cells crosslinked with formaldehyde *in situ*^{15,16}. We then analysed the SIR3-associated DNA by using the polymerase chain reaction (PCR) with gene-specific primers. Sequences from *HMLα* and telomeric *ADH4* (ref. 17) can be detected in a cross-linking and SIR3HA-dependent manner (Fig. 2a, lanes 2–4). These sequences are 10- to 20-fold enriched over *PHO5*, *GAL1* and *ACT1* sequences, which are more than 50 kilobases away from telomeres. The silent *HMRα* locus and telomeric *URA3* (*URA3Tel*)¹⁷ show a similar enrichment over the active *MATA* locus and *URA3* at its normal chromosomal location (Fig. 2b, lane 3). By using antibodies to RAP1 and SIR4 we have found that these factors are also associated with the silent

FIG. 2 a, Crosslinking-dependent immunoprecipitation of silent chromosomal regions. SIR3HA was immunoprecipitated with or without prior crosslinking, and DNA samples from the WCEs (lanes 5–7), a serial dilution thereof (lanes 8–10), and the immunoprecipitates (lanes 2–4) were analysed by PCR with primer pairs from *HML α* , *PHO5* and *GAL1*, or *ADH4* and *ACT1*. The *ADH4* sequences are about 1.2 kb from the telomere adjacent to and telomere-distal from transcriptionally repressed *URA3* (ref. 17). b, SIR4- and RAP1-dependent association of SIR3 with *HMRa*, *HML α* , *ADH4* and telomeric *URA3*. PCR analyses of immunoprecipitates from strains WT (lane 3) or mutant for SIR4 and RAP1 (lanes 4 and 5). As RAP1–21 de-represses telomeric genes but affects *HML α* sequences only partially¹⁴, *HML α* sequences are still slightly enriched. c, H4 mutations affect co-immunoprecipitation of SIR3 with *HML α* and telomeric regions. Chromosome V-R primers are located 12.5, 17.5 and 20.5 kb from the telomere, beyond the X and Y' repeats; chromosome VI-R primers are located at 2.5, 5.0, 7.5 and 15.0 kb from the telomere. M, size standard. Lane 1, no DNA in the PCR mix. METHODS. WCEs from formaldehyde crosslinked cells¹⁵ were prepared in 50 mM HEPES/KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate, and protease inhibitors. Chromosomal DNA was sheared by sonication to an average size between 0.5 and 1.0 kb. SIR3HA-immunoprecipitates were washed with lysis buffer; lysis buffer/500 mM NaCl; 10 mM Tris/HCl, pH 8.0, 0.25 M LiCl, 0.5% NP-40, 0.5% Na-Deoxycholate, 1 mM EDTA; and finally TE. After DNA purification¹⁵, PCR reactions²³ (25 cycles) used 1/50 of the immunoprecipitates, 1/13,500 of the inputs, and serial 2.5-fold dilutions of these as templates. PCR products were separated on a 6% PAG, and visualized with ethidium bromide. Photoprocessing was performed using the OFOTO software (Light Source Computer Images).

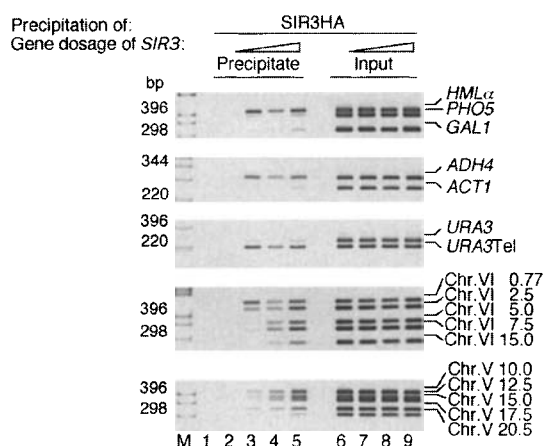
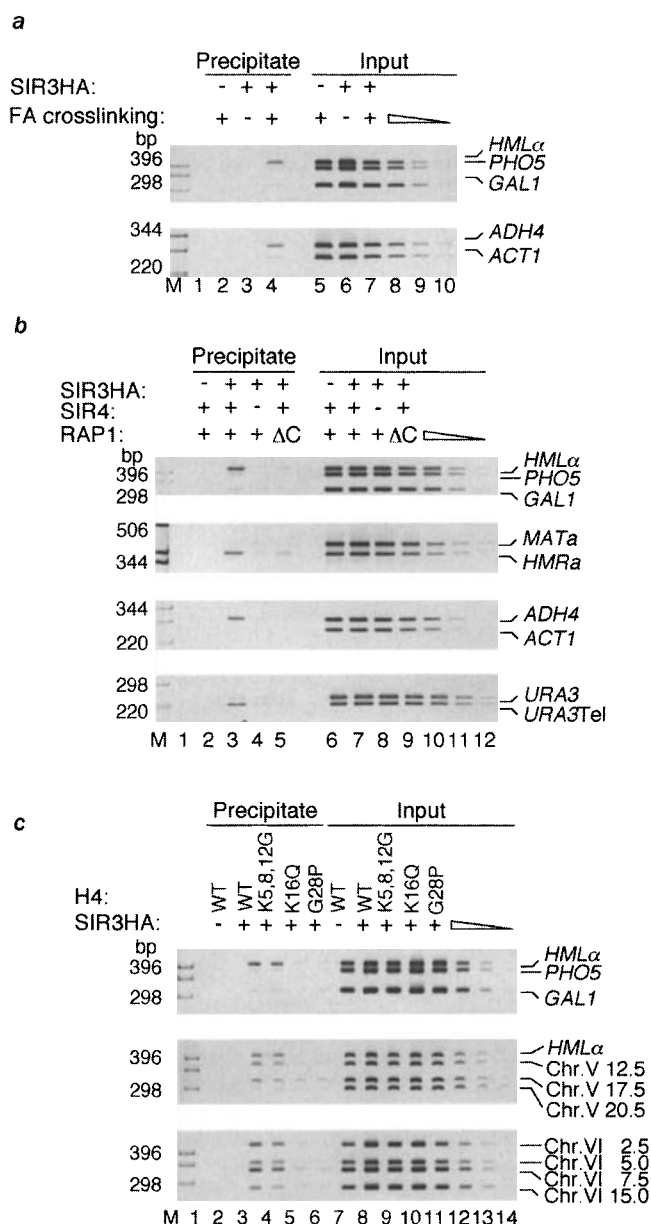


FIG. 3 Increased intracellular levels of SIR3 cause the spreading of SIR3 into telomere-distal chromatin. Epitope-tagged SIR3HA was precipitated from formaldehyde crosslinked WCEs prepared from strains expressing increasing amounts of SIR3. Representation of the sequences from *HML α* , *PHO5*, *GAL1*, *ADH4*, *ACT1*, telomeric and internally located *URA3* and telomeric regions of chromosomes V-R and VI-R in the input materials or immunoprecipitates was analysed by PCR. Chromosome V-R was previously used to demonstrate SIR3 dosage-dependent spreading of telomeric silencing¹²; M, molecular weight markers. Lane 1, no template DNA in the PCR mix; input materials (lanes 6–9) and precipitates (lanes 2–5) from strains with WT SIR3 (lane 2), expressing SIR3HA from a genomic copy of the gene (lane 3), from a CENARS plasmid (lane 4), from a 2 μ plasmid (lane 5). METHODS. WT SIR3 or SIR3HA were reintegrated into AYH2.8, respectively, by targeting *SIR3*–*HIS3* or *SIR3HA*–*HIS3* cassettes to the *sir3*::*LEU2* allele and isolation of His⁺/Leu⁺ transformants. Relative abundance of SIR3 as judged from western blots is 1:4:10 (genomic copy:CENARS:2 μ). Primers for chromosomes V-R and VI-R are located at 10.0–20.5 kb and 0.77–15 kb, respectively.

loci in a manner similar to that of SIR3 (S.S.-B. A.H., and M.G., unpublished data). That SIR3 is not exclusively present in silent chromosomal regions (especially when overexpressed) might result from its ability to interact with histones H3 and H4 (ref. 13), which are present throughout the genome, thus leading to low frequency or transient interactions. Nonetheless, SIR3 is a chromosomal protein preferentially found at *HMRa*, *HMLα* and near a telomere *in vivo*.

We next investigated the way these interactions are affected by mutations in SIR4, RAP1 and histone H4. In strains lacking SIR4 or carrying the silencing-defective *RAP1-21* allele, the interaction of SIR3 with *HMRa*, *HMLα* or *URA3Tel* and *ADH4* is greatly decreased (Fig. 2b, lanes 4 and 5). SIR4 and RAP1 seem to be required for the silencing-relevant recruitment of SIR3 to chromatin or the stabilization of its chromosomal interactions *in vivo*. It is therefore surprising that the absence of SIR4 had only a minor effect on complex formation in the absence of crosslinking (Fig. 1a, lane 3). This difference might be due to the higher stringency (salt, detergent) of the crosslinking protocol. Nevertheless, the interactions between SIR3 and chromatin in unfixed extracts are likely to be physiologically significant in that they are still sensitive to RAP1 and H4 mutations which prevent silencing.

Single amino-acid substitutions in histone H4 also abolish the interaction of SIR3 with *HMLα* and with sequences from the telomeres on the right arm of chromosome V (V-R) or the right arm of chromosome VI (VI-R) (Fig. 2c, lanes 5 and 6). The triple amino-acid exchange K5, 8, 12G has no obvious effect (Fig. 2c, lane 4). We conclude that the crosslinking/PCR analyses accurately reflect the silencing phenotypes of the H4 mutants and confirm the importance of the H4 N terminus for the binding of SIR3 to chromatin *in vivo*.

In cells with wild-type levels of SIR3, genes near telomeres are subject to silencing only within a region of about 2–4 kb from the telomere¹². Elevating the amount of SIR3 present causes an expansion of this silent region up to 16 kb or more at the telomere of chromosome V-R¹². However, the frequency of silencing decreases with greater distances from the telomere. We wished to see whether spreading of the transcriptionally silent state is accompanied by a physical spreading of SIR3 along the chromosome. We found that the amounts of telomere-distal DNA sequences precipitated from chromosomes V-R or VI-R increase when the gene dosage for SIR3 is increased (Fig. 3, lanes 3–5, and Fig. 4). Enhanced interactions with SIR3HA can be measured up to at least 17.5 kb from the end of chromosome V-R, whereas the amount of DNA precipitated by SIR3HA at 20.5 kb and 30.0 kb is similar to that of genes distant from the telomere, such as *ACT1* on chromosome VI (Fig. 4). Regions closer than 10.0 kb to the chromosome V-R telomere were not analysed because of the presence of the repetitive X and Y' elements. At chromosome VI-R, SIR3 spreads up to at least 15.0 kb (Figs 3 and 4). In contrast, the representation of *HMLα*, *ADH4*, *URA3Tel* or the 0.77-kb region of ChrVI-R does not increase when SIR3HA is overexpressed (Fig. 3, compare lanes 3 and 5); this has been confirmed by scanning and quantifying the DNA bands (data not shown). Therefore SIR3 is present at the HM loci and very close to telomeres at a similar level even when SIR3 is overexpressed. In contrast, higher levels of SIR3 allow its histone-dependent spreading into telomere-distal chromatin. The amount of SIR3-associated DNA decreases with greater distances from the telomere, correlating with the extent of silencing (Fig. 4). SIR3 precipitates slightly more DNA at chromosomes VI- and V-R at distances of 7.5 kb and 17.5 kb, respectively, from the telomeres. We cannot exclude the possibility that there are minor internal foci which bind SIR3 more efficiently. Alternatively, the increase may be the result of more efficient PCR reactions with primers at these sites as these sites at chromosomes VI-R and V-R are also more abundant in the input material (Figs 2c and 3).

Heterochromatin-mediated gene repression is widespread among eukaryotes¹⁸. A general concept emerging for the formation of heterochromatin is that specific DNA elements induce the

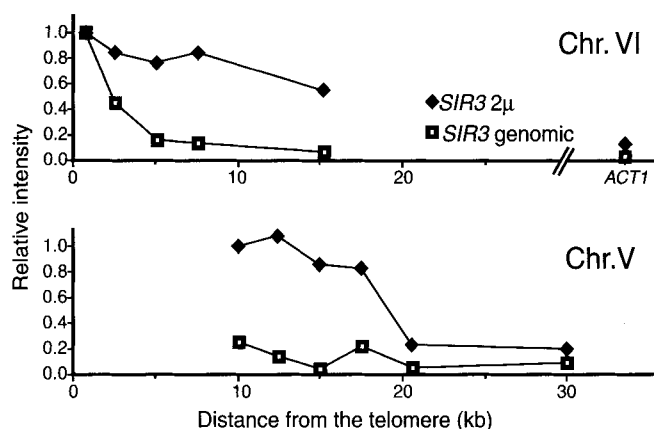


FIG. 4 Relative abundances of subtelomeric regions from chromosomes VI-R (top) and V-R (bottom) immunoprecipitated by SIR3HA from strains expressing increasing amounts of SIR3HA from a genomic copy of its gene, or a multicopy 2 μ plasmid.

METHODS. Ethidium bromide-stained PCR products were photographed on Polaroid 55 film, scanned and quantified using NIH Image1.49 software. Band intensities in the immunoprecipitates were normalized according to the relative intensities in the input materials to correct for PCR-induced variations. At chromosomes VI-R the 0.77-kb region was assigned the relative abundance 1.0; at chromosome V-R the 10.0-kb region was taken as reference point. Average values from at least three experiments are shown.

conversion of adjacent chromatin into a repressive structure. In yeast, telomeres with RAP1 bound to them may serve as initiator elements from which chromosomal complexes containing SIR3 originate^{6,7,13}. Both RAP1 and H4 seem to be necessary for the initial association of SIR3 with chromatin. This interdependence may reduce random interactions of SIR3 with histones H3 and H4 present throughout the genome, and favour the recruitment of SIR3 to specific chromosomal regions. When nucleation occurs, SIR3 might then polymerize along the chromosome, possibly in a cooperative fashion. The correlation between the presence of SIR3, the spreading of the silent state of chromatin, and, where analysed, the refractive nature of silent chromatin^{19,20}, suggest that SIR3 promotes silencing by condensing chromatin as it interacts with the H3 and H4 N termini. The histone dependence of heterochromatin in *Drosophila melanogaster*²¹ suggests that similar principles may underlie the formation of heterochromatin in other eukaryotes. □

Received 3 May; accepted 5 July 1996.

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ACKNOWLEDGEMENTS. We thank D. Gottschling, A. Lustig and F. Lenfant for plasmids; L. Johnson for yeast strains; A. Carmen for anti-histone antibodies; and G. Fieser for the monoclonal antibody 17D09. This work was supported by a Public Health Service grant from the NIH to M.G. S.S.-B. received a fellowship from the Deutsche Forschungsgemeinschaft.

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Release of both native and non-native proteins from a *cis*-only GroEL ternary complex

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PROTEIN folding by the double-ring chaperonin GroEL is initiated in *cis* ternary complexes, in which polypeptide is sequestered in the central channel of a GroEL ring, capped by the co-chaperonin GroES^{1–3}. The *cis* ternary complex is dissociated (half-life of ~15 s) by *trans*-sided ATP hydrolysis, which triggers release of GroES^{4,6}. For the substrate protein rhodanese, only ~15% of *cis*-localized molecules attain their native form before hydrolysis^{2,7}. A major question concerning the GroEL mechanism is whether both native and non-native forms are released from the *cis* complex. Here we address this question using a '*cis*-only' mixed-ring GroEL complex that binds polypeptide and GroES on only one of its two rings. This complex mediates refolding of rhodanese but, as with wild-type GroEL, renaturation is quenched by addition of mutant GroEL 'traps', which bind but do not release polypeptide substrate^{7,8}. This indicates that non-native forms are released from the *cis* complex. Quenching of refolding by traps was also observed under physiological conditions, both in undiluted *Xenopus* oocyte extract and in intact oocytes. We conclude that release of non-native forms from GroEL *in vivo* allows a kinetic partitioning among various chaperones and proteolytic components, which determines both the conformation and lifetime of a protein.

To produce a '*cis*-only' GroEL complex, which is able to bind polypeptide and GroES on only one and the same ring, we formed a mixed-ring GroEL complex, in which one ring was composed of wild-type subunits and the other of subunits with apical domain substitutions that prevent both polypeptide and GroES binding (Fig. 1a). In particular, the Y203E substitution abolishes binding of both polypeptide and GroES⁸ and the pair of substitutions G337S/I349E cause a change in migration compared with wild-type GroEL on anion-exchange chromatography⁷, in addition to affecting GroES binding⁸. Note that a ring with substitutions at positions 203/337/349 (termed 3-7-9) should be defective in apical domain function, whereas the unaltered equatorial ATP-binding domains should be fully functional for ATP hydrolysis and in signalling polypeptide and GroES binding and release from an adjoining wild-type ring^{8–10}.

A mixed-ring complex was formed by co-incubation of purified wild-type complex with 3-7-9 complex at 42 °C in the presence of Mg-ATP. When the mixture was fractionated by anion-exchange chromatography at 23 °C, a peak appeared between the migration peaks of the parent complexes (Fig. 1b). The central portion of this peak migrated as a single peak of the same mobility on rechromatography, reflecting its stability (not shown). When analysed by gel filtration, the isolated product migrated as wild-type GroEL, indicating that it had a double rather than single ring (not shown). Because subunits bearing the 3-7-9 substitutions migrate in SDS-PAGE more slowly than wild-type subunits, the subunit composition of the isolated complex could be determined. Equal amounts of wild-type and 3-7-9 subunits were observed (Fig. 1b), consistent with the formation of a 1:1 mixed-ring complex. In support of our conclusion that two homo-oligomeric rings were present, as opposed to two rings with hetero-oligomeric composition, we found that when disproportionate amounts of the two input 'parental' homo-oligomers were mixed in ATP at 42 °C, there was still a 1:1 ratio of wild-type and 3-7-9 subunits in the central peak (not shown). If hetero-oligomeric rings had been formed, the relative amounts of wild-type and 3-7-9 subunits in reassembled rings should have reflected the disproportion of the input parent complexes.

We examined the binding of GroES and substrate polypeptide by the isolated mixed-ring complex, called MR1, to determine whether these ligands were recognized only by the wild-type ring. First, a binary complex between MR1 and GroES was formed in the presence of ADP, a condition in which GroES forms a stable asymmetrical complex, binding at only one end of the GroEL cylinder^{11,12}. To investigate whether the wild-type GroEL ring was selectively bound, we made use of an earlier observation¹¹ that the flexible carboxy termini of GroEL subunits are cleaved by proteinase K added in the absence of GroES, but that binding of GroES protects subunits of the bound GroEL ring from cleavage. When a wild-type GroEL–GroES–ADP mixture was treated with proteinase K, half of the GroEL subunits were susceptible to digestion (Fig. 2a, lanes 3 and 4). By contrast, when the MR1–GroES mixture was treated with proteinase K (Fig. 2a, lane 2), wild-type GroEL subunits were completely protected, as shown by failure to produce any faster-migrating product (compare with lane 4), indicating that GroES had quantitatively occupied the wild-type ring (Fig. 2a). The 3-7-9 subunits in the other ring presumably were cleaved, but the smaller product could not be detected because it migrates with uncleaved wild-type subunit (not shown). There was also some abnormal internal cleavage in the 3-7-9 subunits (asterisk in Fig. 2a), probably within the altered apical domains.

Next, a binary complex was formed between the MR1 complex and rhodanese molecules bearing an ¹²⁵I-radiolabelled cross-linker, APDP, which is photoactivatable and can be cleaved^{1,7}. The binary complex was photolysed, the crosslinker released from rhodanese by reduction, and the products fractionated by SDS-PAGE. Specific radiolabelling of wild-type but not 3-7-9 subunits was observed (Fig. 2b), indicating that rhodanese was bound specifically by the ring containing wild-type subunits.

Although only the apical domains of the wild-type ring in MR1 can interact with the polypeptide and GroES ligands, the equatorial domains of both rings were presumed to function normally. This was supported by the equivalent ATP hydrolysis by equal amounts of MR1 and wild-type complexes (not shown). Addition of GroES also produced identical partial inhibition of ATPase activity^{5,13–16}.

The function of MR1 in refolding rhodanese was next investigated. Binary complexes of rhodanese–MR1 or rhodanese–GroEL, containing equal amounts of rhodanese, were individually incubated with GroES and Mg-ATP, and the time course of rhodanese reconstitution determined (Fig. 3a). For both reactions, the *t*_{1/2} for refolding was ~8 min, and, for both complexes, refolding was essentially complete by 20 min. The MR1 complex was notably stable through the course of the reaction, as deter-

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mined by anion-exchange chromatography (not shown). We conclude that the kinetics of folding of the *cis*-only complex are virtually the same as wild-type GroEL. This suggests that initial production of a *trans* ternary complex with wild-type GroEL and conversion to the productive *cis* complex is not a rate-limiting step

in chaperonin-mediated folding, consistent with observations that polypeptide folding and GroES release are the slow steps in the reaction^{5,7}.

To test whether non-native forms of rhodanese were released from the MR1 complex during the folding reaction, we measured

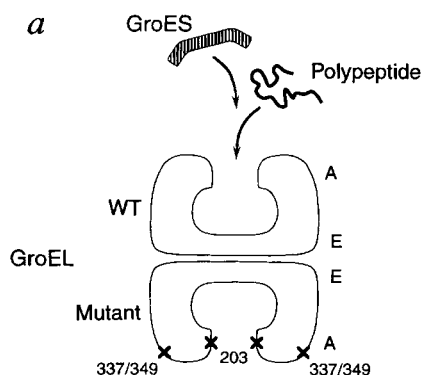
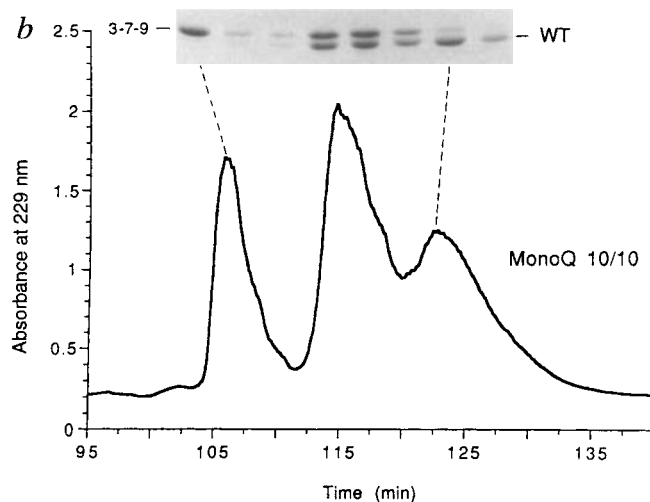


FIG. 1 Formation and characterization of a mixed-ring GroEL complex, MR1, in which only one of the two rings can bind GroES and substrate polypeptide. **a**, Diagram of MR1. One ring is composed of wild-type (WT) subunits and the other contains three apical mutations in each subunit: Y203E, affecting a residue facing the central channel, which prevents binding of both substrate polypeptide and GroES; and the pair of mutations, G337S and I349E, in a pair of α -helices mapping to the outer aspect of the domain, which confer a migration distinct from wild-type GroEL during anion-exchange chromatography and affect GroES binding⁸. A, E: apical and equatorial domains, respectively. **b**, Purification of MR1 mixed-ring complex from homotetradameric wild-type and mutant 203/337/349 (3-7-9) complexes using anion-exchange chromatography. Fractions across the GroEL peaks were analysed by SDS-PAGE and Coomassie blue staining.

METHODS. Y203E/G337S/I349E (3-7-9) mutant complex was produced from a GroEL expression plasmid⁸. To form MR1, 5 mg each of wild-type



GroEL and 3-7-9 were mixed in 10 ml buffer A (50 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM MgCl₂, 5 mM ATP) and incubated at 42 °C for 45 min. The mixture was cooled to 23 °C, CDTA was added to 10 mM, and the sample was fractionated on a MonoQ 10/10 anion-exchange column (Pharmacia) with a NaCl gradient in 50 mM Tris-HCl, pH 7.4. Fractions were collected and analysed in SDS-PAGE (10%). The central MR1 peak, containing equal amounts of 3-7-9 and wild-type subunits, was pooled, and an aliquot rechromatographed; MR1 eluted as a single peak (not shown), indicating the stability of MR1 and the absence of any contaminating homotetradecamers.

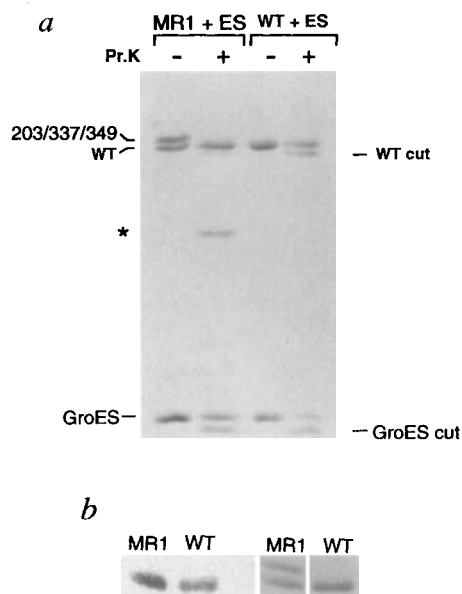


FIG. 2 GroES and non-native substrate protein bind to the wild-type ring of MR1. **a**, GroES binds to the wild-type ring of MR1 in the presence of ADP, protecting the termini of wild-type subunits from digestion by proteinase (Pr.K) SDS-PAGE shows wild-type and 3-7-9 mutant GroEL subunits, GroES, and the products of proteolytic digestion, detected by Coomassie staining. Asterisk designates a product of *M*_r ~40K, presumed to be derived from internal cleavage of 3-7-9 subunits. **b**, Non-native rhodanese bearing an ¹²⁵I-labelled, photoactivatable, cleavable crosslinker *N*-[4-(*p*-azidosalicylamido)butyl]-3'-(2'-pyridyldithio)propionamide (APDP) which selectively labels the wild-type ring of MR1. Mutant and wild-type GroEL subunits were detected by Coomassie staining (right panel), and the radiolabelled subunits by phosphorimaging (left panel) after SDS-PAGE.

METHODS. **a**, Binary complexes between either MR1 or WT GroEL and GroES were formed by mixing 285 nM MR1 or WT GroEL oligomer with 1.7 μ M GroES oligomer (in 25 mM MOPS, pH 7.2, 50 mM KCl, 5 mM MgCl₂ and 5 mM ADP) and incubating for 20 min at room temperature. Complexes were digested with 480 ng ml⁻¹ proteinase K for 8 min before quenching with 1 mM phenylmethylsulphonylfluoride (PMSF). Samples were separated on SDS-PAGE (10%) and detected using Coomassie stain. **b**, ¹²⁵I-labelled APDP-rhodanese was prepared as described⁷, except that rhodanese was unfolded using 8 M urea. Binary complexes of ¹²⁵I-APDP-rhodanese with MR1 or WT GroEL were formed by rapidly diluting unfolded rhodanese 100-fold into 0.4 mg ml⁻¹ MR1 or WT GroEL in buffer A, then exposed to UV light at 350 nm for 5 min to activate the crosslink. Rhodanese was released by reduction with DTT in SDS-sample buffer, and the proteins separated by SDS-PAGE.

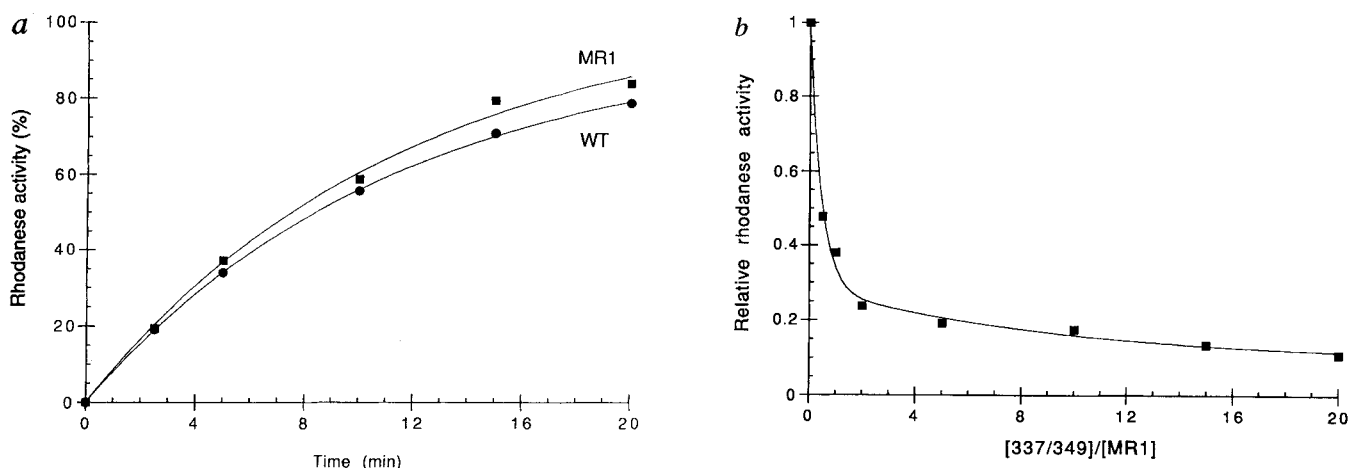


FIG. 3 Kinetics of refolding of rhodanese in the presence of MR1 are the same as in the presence of WT GroEL, and the folding reaction can be quenched by addition of mutant GroEL traps. *a*, In the presence of Mg-ATP and GroES, rhodanese refolds with the same rate constant and efficiency with MR1 (squares) as with wild-type GroEL (circles). *b*, Rhodanese refolding in the presence of MR1 can be quenched by the addition of an excess of a mutant-GroEL trap (337/349), which is able to bind non-native polypeptides but not release them, demonstrating that non-native forms of rhodanese are released from MR1 *cis* ternary complex before folding to the native state is complete.

METHODS. Binary complexes with WT GroEL and MR1 were made as before⁷, except that rhodanese was denatured in 7 M urea. MR1-rhodanese binary complex was incubated at 4 °C for 12 h to ensure exclusive binding to the wild-type ring, as confirmed by photocrosslinking (Fig 2b and data not

shown). Binary complexes were purified by anion exchange⁷. To monitor the integrity of the MR1 complex, a folding reaction was done by adding 4 μ M GroES to 1.4 μ M MR1-rhodanese binary complex in buffer A with 10 mM DTT and 5 mM Na₂S₂O₃. After 40 min at 25 °C, the reaction was quenched with 10 mM CDTA and chromatographed as before. The MR1 complex eluted as a single peak, confirming its stability under these refolding conditions. *a*, The kinetics of refolding of rhodanese at 25 °C from WT GroEL and MR1 were monitored by adding 3 μ M GroES to 1 μ M binary complex in buffer A with 10 mM DTT and 5 mM Na₂S₂O₃. At the indicated times, samples were withdrawn and assayed for rhodanese enzymatic activity at 37 °C (ref. 23). *b*, Rhodanese refolding reactions were carried out as described, except that the 337/349 mutant GroEL trap was included at the indicated ratios to MR1, and the extent of folding was determined after 20 min.

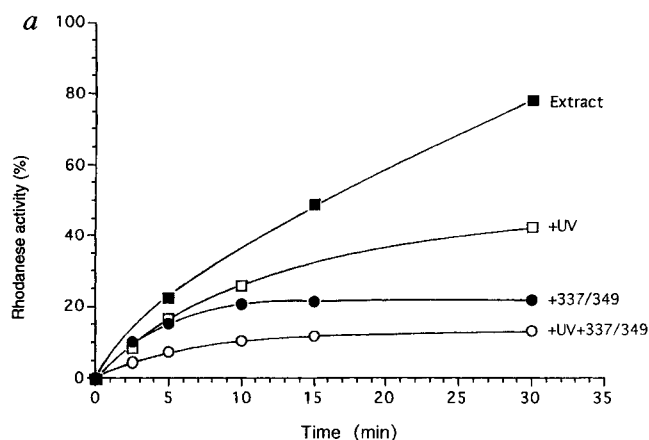


FIG. 4 Rhodanese refolding and release of non-native forms from binary complex with GroEL occurs in the physiological setting of undiluted *Xenopus* oocyte extract and intact *Xenopus* oocytes. *a*, Rhodanese refolds efficiently from binary complex in *Xenopus* oocyte extract, but refolding can be inhibited by the presence of either endogenous or exogenous traps. The kinetics of rhodanese refolding from GroEL-rhodanese complex in the presence of GroES and ATP were determined in undiluted *Xenopus* oocyte extract (solid symbols) and in ATP-crosslinked *Xenopus* oocyte extract (open symbols), in either the absence (squares) or presence (circles) of added 337/349 mutant GroEL trap. *b*, Recovery of rhodanese activity from binary complex injected into intact *Xenopus* oocytes is high, but is inhibited by co-injection of a mutant GroEL trap. The yield of rhodanese activity from GroEL-rhodanese binary complex and GroES injected into intact *Xenopus* oocytes either alone (left), with a 10-fold excess of 337/349 GroEL mutant trap (middle), or with a 10-fold excess of wild-type GroEL (right) was determined after 30 min of refolding. EL, GroEL; ES, GroES.

METHODS. *a*, Undiluted *Xenopus* oocyte extract, prepared as described¹⁸, contains ~100 mg ml⁻¹ endogenous proteins, added protease inhibitors, Mg-ATP, creatine phosphate and cytochalasin B. To produce ATP-cross-linked extract¹⁹, the extract was irradiated at 254 nm with 900 mJ over 25 min in a Stratilinker UV crosslinker (Stratagene), supplemented with a further 1 mM ATP, and exposed to the same UV treatment. Refolding reactions were carried out by adding 5 mM ATP and 4 μ M GroES, with or without 12 μ M 337/349 trap, to the extract and then adding WT GroEL-rhodanese binary complex to 2.4 μ M. Samples were withdrawn at the indicated times, and assayed for rhodanese activity²³. *b*, For each reaction, 100 *Xenopus* oocytes were injected with 40 nl buffer containing 0.25 μ g WT GroEL-rhodanese binary complex and 0.1 μ g GroES, either alone or with 2.5 μ g 337/349 mutant GroEL trap, or with 2.5 μ g WT GroEL. After 30 min at 25 °C, cells were homogenized in 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM EDTA (2 μ l per oocyte) to quench folding, the homogenate was centrifuged at 15,000g for 15 min, and the supernatant assayed for rhodanese activity²³. Results are corrected for a small amount of background activity (<10%).

the effect of a trap version of GroEL on rhodanese refolding. The 337/349 tetradecamer, able to bind but not release polypeptide in the presence of GroES and Mg-ATP, was used^{7,8}. At a 1:1 molar ratio of 337/349 to MR1, regained activity was reduced to 40% (Fig. 3b), and at a molar ratio of 2:1 or greater, recovery was ~20%, comparable to previous observations with wild-type GroEL–rhodanese, where rhodanese refolding was similarly quenched and rhodanese was transferred to the 337/349 trap⁷. These results with MR1 indicate that not only native but also non-native forms are released from the *cis* channel of GroEL.

It has been suggested that release of non-native forms is an artefact of *in vitro* experiments carried out in dilute aqueous buffers and that under intracellular conditions macromolecular crowding would prevent such release¹⁷. To assess whether release of non-native forms from GroEL can occur under physiological conditions, purified rhodanese–GroEL binary complex was introduced either into *Xenopus* oocyte extract or into intact oocytes, and recovery of activity was examined in the absence or presence of trap molecules. For extract studies, intact oocytes were centrifuged without added buffer¹⁸ to remove membranes and marginated yolk platelets, producing an undiluted supernatant (total protein ~100 mg ml⁻¹). Binary complex was added after GroES and Mg-ATP. We observed ~80% recovery of rhodanese activity during 30 min at room temperature (Fig. 4a, extract) in a reaction dependent on GroES (not shown) and slower than in buffer solution. When 337/349 trap was added at a 5:1 ratio, recovery was reduced to ~20%, with inhibition apparent by 5 min (Fig. 4a, +337/349). We asked whether endogenous chaperone components in the oocyte extract could also function as 'traps', because in a control study rhodanese diluted from urea into extract could refold in an ATP-dependent fashion without exogenous GroEL/GroES (not shown); if nucleotide could be crosslinked to such chaperones, they might bind non-native rhodanese but be unable to release it, functioning as endogenous 'traps'. Accordingly, we supplemented the oocyte lysate with 1 mM ATP and irradiated it with ultraviolet light to crosslink bound nucleotide¹⁹. When rhodanese–GroEL was added to lysate treated in this way, and GroES and more ATP added, refolding was quenched to ~50% of that for untreated lysate (Fig. 4a, +UV). The effects of endogenous traps and exogenous 337/349 trap were partially additive, reducing refolding to ~10% when 337/349 was present in a ultraviolet-treated lysate (Fig. 4a, +337/349+UV). We conclude that rhodanese can be released from GroEL in non-native form in a physiological mixture and that the non-native form can partition between both other GroEL molecules and other component(s).

To assess whether release occurs similarly in an intact cell, rhodanese–GroEL binary complex was injected into intact *Xenopus* oocytes in the absence or presence of 337/349 trap; after 30 min, the cells were lysed in the presence of EDTA to quench any further chaperone-mediated refolding, and rhodanese activity assayed. In the absence of trap, 70% of rhodanese activity was recovered (Fig. 4b). In the presence of trap, however, only 20% of activity remained. By contrast, when an identical amount of wild-type GroEL was co-injected instead of trap, recovery was ~60%. We conclude that even in the milieu of an intact cell, rhodanese can be released from GroEL in non-native forms that can undergo kinetic partitioning. In summary, ternary GroEL–GroES–polypeptide complex commits a fraction of substrate polypeptide to the native state *in vivo* during each chaperonin cycle, while the remaining released non-native forms have the opportunity to partition to other chaperones^{20,21} or proteolytic components²². Such partitioning probably acts as a major determinant of the fate of non-native proteins in the living cell. □

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ACKNOWLEDGEMENTS. S.G.B. and J.S.W. made equal contributions to this work. We thank S. Sondak and L. Scharl for technical assistance. The work was supported in part by grants from the NIH to A.L.H. and W.A.F. S.G.B. is the recipient of a Wellcome Trust Travelling Research Fellowship.

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Role of CBP/P300 in nuclear receptor signalling

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THE nuclear receptor superfamily includes receptors for steroids, retinoids, thyroid hormone and vitamin D, as well as many related proteins^{1–3}. An important feature of the action of the lipophilic hormones and vitamins is that the maintenance of homeostatic function requires both intrinsic positive and negative regulation^{4,5}. Here we provide *in vitro* and *in vivo* evidence that identifies the CREB-binding protein (CBP) and its homologue P300 (refs 6, 7) as cofactors mediating nuclear-receptor-activated gene transcription. The role of CBP/P300 in the transcriptional response to cyclic AMP, phorbol esters, serum, the lipophilic hormones and as the target of the E1A oncoprotein suggests they may serve as integrators of extracellular and intracellular signalling pathways.

The retinoid X receptors (RXRs) and their heterodimeric partners possess a characteristic DNA-binding domain followed by a carboxy-terminal ligand-binding domain (LBD) which is a complex region that includes subdomains for dimerization functions, transcriptional activation and, in some cases, transcriptional repression (Fig. 1a). Negative transcriptional regulation by the thyroid-hormone receptor (TR) and the retinoic acid receptor (RAR) is mediated, in part, by their association with a class of silencing mediators termed SMRT and N-CoR (refs 8, 9). In addition, at least three distinct classes of receptor cofactors have been identified that include SRC1, TRIP1, RIP140/160 and TIF1 (refs 10–15). We and others have shown that nuclear receptors may 'cross-couple' with AP-1 (Jun/Fos)¹⁶ and CREB (D.C., unpublished results), which use the CREB-binding protein (CBP) as a cofactor (Fig. 1a)⁶. Microinjection of neutralizing

Received 3 May; accepted 28 June 1996.

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antibodies against CBP into cell nuclei completely blocks CREB- and AP-1-mediated signalling¹⁷, and signalling to nuclear receptors as well (V.L., unpublished results, and see later), indicating that CBP/P300 may mediate other signalling pathways.

The interaction between CBP and the retinoid receptor RXR is shown in Fig. 1b. A matrix-bound fusion protein of glutathione-S-transferase with RXR(GST-RXR), but not GST alone, retains radiolabelled CBP when ligand is added (lanes 1, 2 and 5, 6). Although phosphorylation of CREB by protein kinase A (PKA) is a prerequisite for its interaction with CBP¹⁷ (lanes 8 and 9), phosphorylation of RXR does not further stimulate CBP association (lanes 3 and 4). Amino- and carboxy-terminal truncation indicates that the LBD is necessary and sufficient for this interaction (lane 7, and data not shown), which is stimulated 5–7-fold by ligand.

The interactions between cellular CBP and nuclear receptors was verified by incubating HeLa cell nuclear extracts with matrix-bound RAR, RXR or RXR/TR in the presence and absence of ligand. Western blot analysis of such affinity-selected proteins show the TR interaction to be more sensitive to ligand (Fig. 1c, lanes 2 and 3) than that of RXR or RAR (lanes 4 and 5, and data not shown), indicating that these receptors can associate with CBP in the presence of a full complement of nuclear proteins. To show that nuclear receptors and CBP/P300 interact *in vivo*, we used a stable cell line that expresses histidine-tagged RXR (I.G.S., unpublished results). Nuclear extracts prepared from these cells retain CBP/P300 on a nickel-affinity column (lane 7), whereas nuclear extracts lacking histidine-tagged RXR fail to retain CBP/P300, as expected (lane 6).

The yeast two-hybrid assay was used to map CBP and receptor interaction domains. Evidence for the interaction between TR and CBP is shown in Fig. 2a. The GAL4 activation domain (GAL-ACT) when fused with receptors is inactive (Fig. 2a–c, first lanes). However, in the presence of the mutant protein Δ 1K3Q, which retains only the amino terminus and the C/H2-bromo domain of

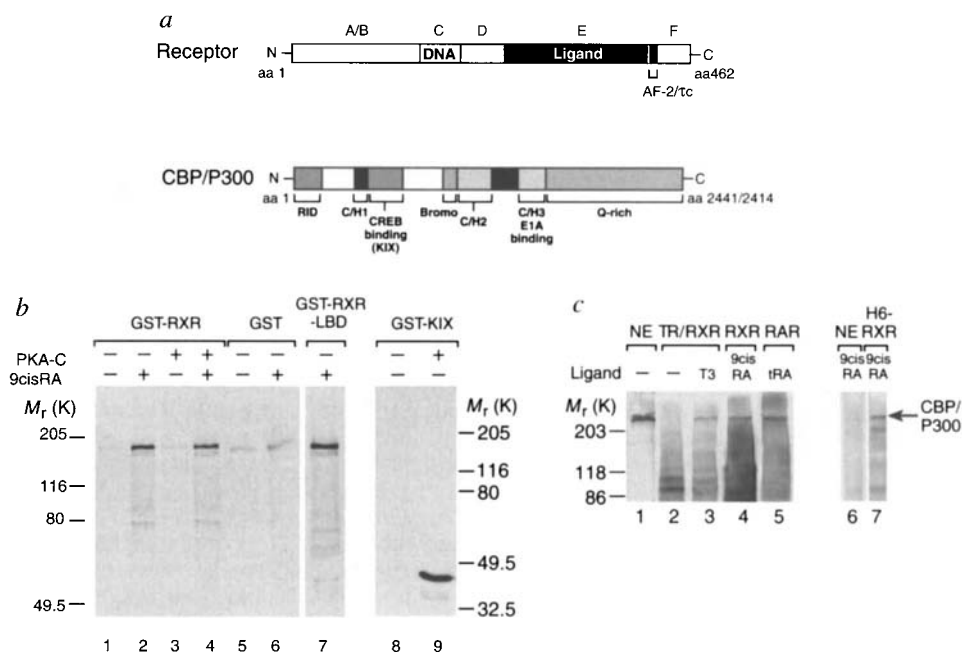
CBP, hormone-dependent interaction is observed with TR–GAL-ACT (Fig. 2a, lane 3). There is a similar interaction between RXR, RAR and CBP (Fig. 2b–c respectively), although in these cases association is only partially stimulated by ligand (Fig. 2b and c, lanes 3). The C/H1 domain shows some interaction with RXR (Fig. 2b, lane 2), but the carboxy-terminal Q-rich region does not (Fig. 2a–c, lane 4). The lack of association of the TR and RAR with the C/H1 region may reflect a true negative interaction or a requirement for additional flanking sequences. Results shown in Fig. 2d indicate that the amino-terminal 170 amino acids constitute the receptor-interacting domain (RID). Far-western blot analysis confirms the *in vivo* results (data not shown). Taken together, our data show that two regions of CBP (that is, RID and C/H1) can interact with nuclear receptor LBDs and perhaps form a bridging contact between RXR and its partners.

Two-hybrid assays in transfected mammalian cells were used to demonstrate ligand-dependent interaction between RXR and CBP. As shown in Fig. 3a, LGD1069, a synthetic RXR-specific ligand, stimulates the interaction between GAL4–CBP and the RXR–LBD–VP16 fusion protein. We also tested the ability of transfected CBP and P300 to stimulate wild-type RXR on a natural reporter gene that is responsive to the RXR homodimer. As shown in Fig. 3b (lane 2), cells transfected with RXR alone show a 2–3-fold increase in reporter activation. When cotransfected, CBP enhances this reporter activation up to 8-fold (lane 4); results were similar with P300 (lanes 5 and 6), although in both cases basal transcription is raised slightly.

A role for CBP/P300 as cofactors in RAR signalling was tested using a heterodimer-specific DR5 (β RE) reporter and a synthetic RAR-specific ligand, AM580. As shown in Fig. 3c, transfection of cells with the reporter alone, or with RAR, results in activation (lanes 1 and 4), which is stimulated 4–5-fold by cotransfection of P300 (lanes 2 and 5) and CBP (data not shown). Transfection of RIP140 (ref. 13), a putative coactivator for the oestrogen receptors, has no effect on this reporter (lanes 3 and 6). As with RXR

FIG. 1 CBP interacts with nuclear receptors *in vitro*.

a, Organization of a typical nuclear receptor and CBP/P300. b, CBP interacts with RXR. ³⁵S-labelled CBP was incubated separately with purified GST (lanes 5 and 6), GST–RXR (lanes 1–4), or GST–RXR–LBD (lane 7) bound to glutathione–Sepharose beads, either in the absence or presence of 1 μ M 9-*cis*-retinoic acid (RA) as indicated. Purified catalytic subunit (C) of protein kinase A and ATP were also included as indicated (lanes 3, 4 and 9). As a positive control, GST fusion of the CREB binding domain of CBP (GST–KIX) was incubated with ³⁵S-labelled CREB (lanes 8 and 9). c, Endogenous CBP interacts with hormone receptors. Crude HeLa cell nuclear extract was allowed to interact with immobilized TR/RXR LBD heterodimer (lanes 2 and 3), RXR (lane 4) or RAR (lane 5), in the presence or absence of appropriate ligands as indicated. T3, thyroid hormone; tRA, all-*trans* retinoic acid. Affinity-selected proteins were separated by SDS–8% PAGE and analysed by immunoblotting using antiserum against CBP. In lane 1 ~200 μ g of crude HeLa cell nuclear extract (NE) was applied to ascertain the position of CBP. Nuclear extracts prepared from HeLa cells (containing CBP/P300 but no histidine-tagged RXR) (lane 6) or CV1 cells stably transfected with histidine-tagged RXR (lane 7) were incubated with Ni–Sepharose beads in the presence of 1 μ M 9-*cis*-RA. Following extensive washing, eluted proteins were immunoprecipitated with antibodies recognizing both CBP and P300 (ref. 17) and protein–A–Sepharose. The immunoprecipitate was separated by SDS–8% PAGE and analysed by western blot using antiserum to P300 (Santa Cruz

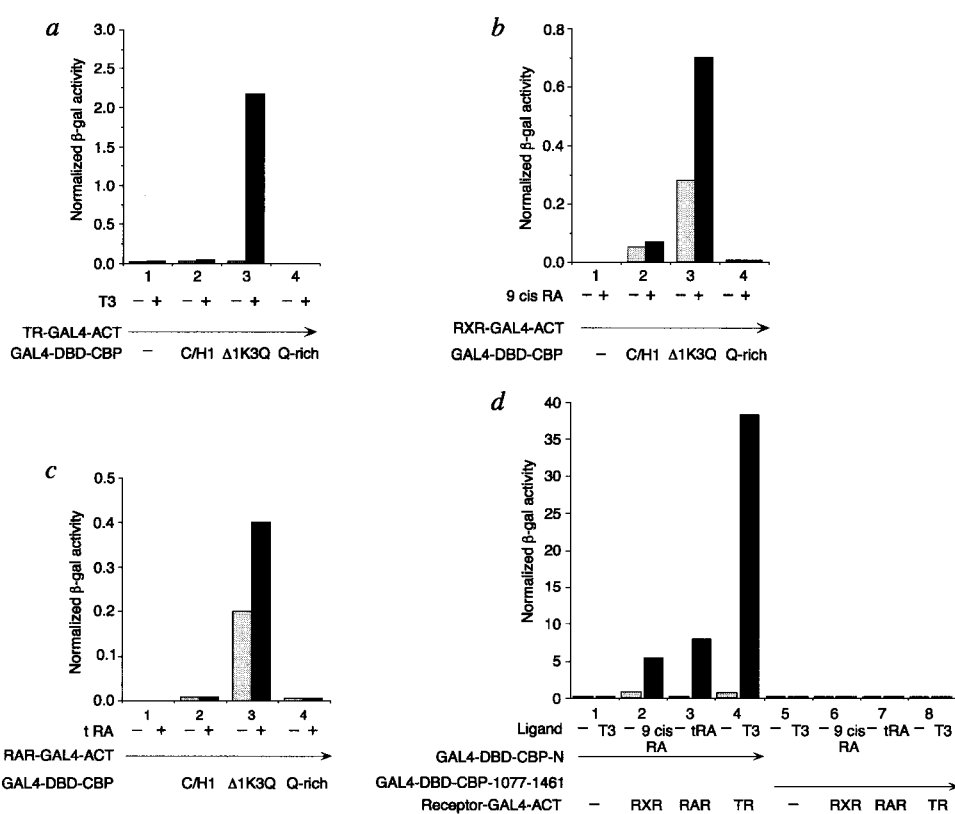


Biotechnology).

METHODS. The construction of GST fusions of RXR, its derivatives, RAR and KIX have been described^{19,20}. Purified GST–TR/RXR LBD heterodimer was a gift from J. W. Schwabe and C. Li. Radiolabelled CBP and CREB were prepared by coupled *in vitro* transcription–translation (Promega) using CMX–CBP and CMX–CREB as template DNA, respectively. CMX–CBP is functional *in vivo* and produces a C-terminal truncated form of CBP when translated *in vitro*⁶.

FIG. 2 CBP interacts with nuclear receptors in yeast. DNAs encoding fusions between the GAL4 activation domain and the ligand-binding domains of TR (TR-GAL4-ACT) (a, d), RXR (RXR-GAL4-ACT) (b, d), and RAR (RAR-GAL4-ACT) (c, d) were introduced into Y190 yeast cells (lanes 1 of a, b and c, respectively) together with DNAs encoding fusions between the GAL4 DNA-binding domain and various isolated domains of CBP (GAL4-DBD-CBP) as indicated. The fusions encode the following regions of CBP: C/H1: amino acids 356–495 (a, b and c, lanes 2); Δ 1K3Q: amino acids 1–116 fused to 720 to 1,459 (a, b and c, lanes 3); Q-rich: amino acids 2,164–2,325 (a, b and c, lanes 4); N: amino acids 1–170 (d, lanes 1–4); and CBP 1077–1461: amino acids, 1,077–1,461 (d, lanes 5–8). β -Galactosidase activity was measured after growth for 14 h in the presence or absence of 100 nM T3 (a, d), 1 μ M 9-cis-RA (b, d), and 1 μ M t-RA (c, d) as indicated.

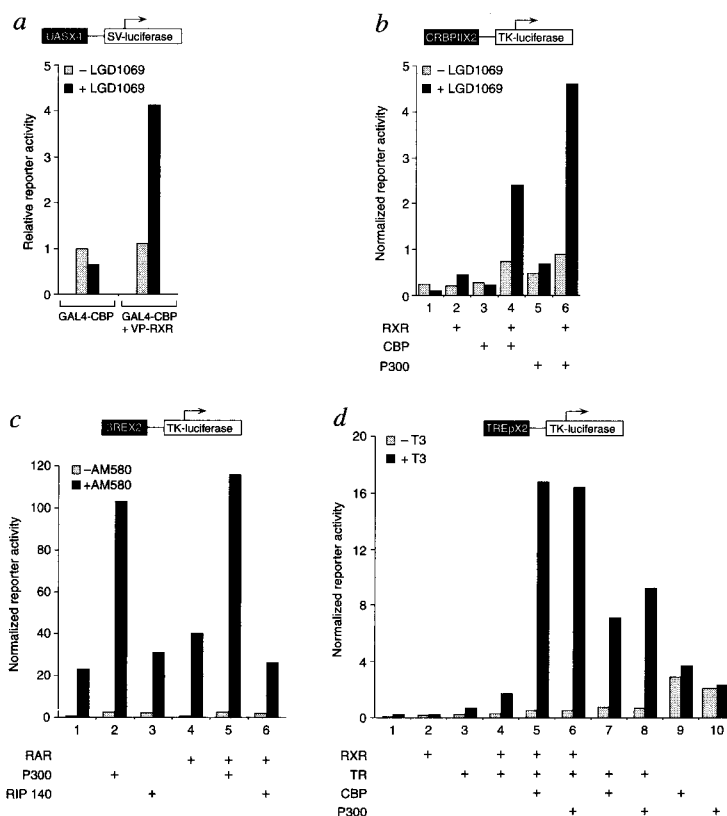
METHODS. Constructions of TR-GAL4-ACT, RXR-GAL4-ACT and RAR-GAL4-ACT have been described¹⁹. GAL4-DBD-CBP derivatives were generated by cloning the following CBP DNA fragments into appropriately digested pGBT9 vector (Clontech): a 425-bp (nt 1,066–1,491) *PvuII*–*PvuII* fragment (C/H1), the N-terminal 1–348 bp *NcoI* fragment fused to a 2,214-bp (nt 2,159–4,373) *EcoRI*–*XhoI* fragment (Δ 1K3Q), a 485-bp (nt 6,488–6,973) *StuI* fragment (Q-rich), a PCR-amplified fragment (510 bp) encoding the N-terminal 170 amino acids (CBP-N), and a PCR-amplified fragment (1,152 bp) encoding amino acids 1,077–1,461 of CBP (CBP-



1077–1461). The construct Δ 1K3Q encodes a GAL4 fusion protein that deletes the C/H1 (1), the CREB-binding domain or KIX (K), the C/H3-E1A binding domain (3) and the Q-rich region of the C-terminus (Q). β -Galactosidase was assayed in permeabilized cells¹⁹.

FIG. 3 CBP/P300 enhance transactivation by nuclear reports. a, Interaction between RXR and CBP is stimulated by ligand in mammalian cells. CV1 cells were transiently transfected with a mixture of DNA containing upstream activating sequence (UAS) $\times$ 4 SV-luc as reporter, CMX- β -gal as an internal control, and expression constructs RSV-GAL4-CBP, and CMX-VP-RXR-LBD where indicated. Relative normalized reporter activity is plotted using the value of luciferase activity of cell extracts transfected with GAL4-CBP without ligand as 1. b–d, CBP/P300 enhance transactivation by RXR, RAR and TR. CV1 cells were transfected with a reporter CRBP11 $\times$ 2 TK-luc (b), β RE $\times$ 2 TK-luc (c), or TRE_p $\times$ 2 TK-luc (d), CMX- β -gal and expression constructs for various proteins as indicated under each experiment. Normalized luciferase activity was determined and plotted as reporter activity. β RE, Retinoid response element present in RAR β gene; TRE_p, palindromic thyroid hormone response element.

METHODS. UAS $\times$ 4 SV-luc was a kind gift from Y. Barak. Constructions of GAL4-CBP, CMX-CBP, CMV-P300 (gift from R. Eckner), pEF-RIP (gift from M. G. Parker), CMX-VP-RXR-LBD, and receptor expression vectors (pCMX-based) have been reported^{6,7,13,21–23}. CV1 cells were plated in 48-well plates and transfected with the indicated DNA mixture by lipofection (DOTAP, Boehringer–Mannheim). DNA used for transfection of 1.6×10^5 cells was: reporter (200 ng), CMX β -gal (500 ng), RSV-GAL4-CBP (160 ng), CMX-VP-RXR-LBD (400 ng), CMX-RXR (50–100 ng), CMX-RAR (20 ng), CMX-rTR α (50 ng), CMX-CBP (200–400 ng), CMV-P300 (100–400 ng), pEF-RIP (100 ng). After transfection, cells were washed twice with PBS and treated with phenol-red-free DMEM with 10% FBS, either alone or with 100 nM ligand (LGD1069 in a and b; AM580 in c; T3 in d) for 40 h before assay²¹. All transfections were done in triplicate.



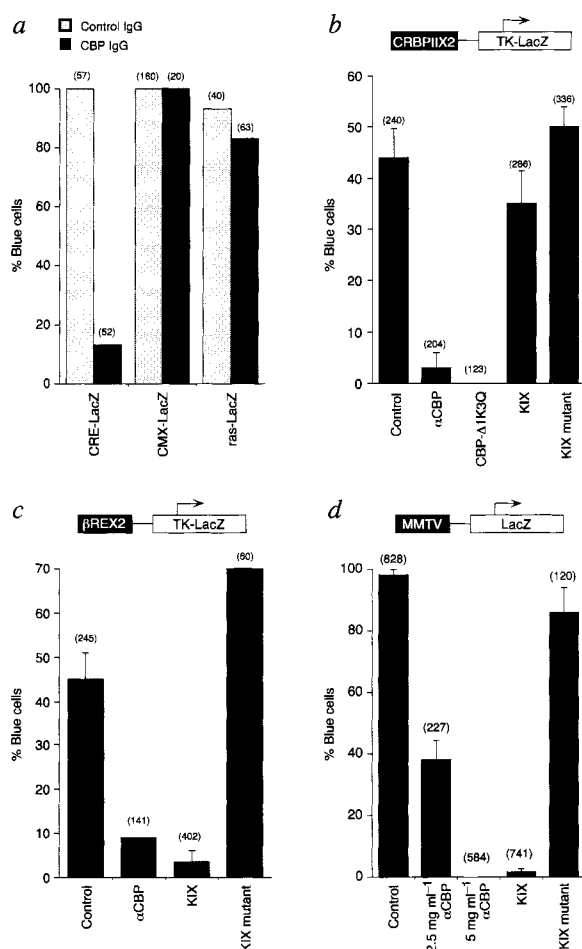


FIG. 4 Endogenous CBP is required for retinoid- and glucocorticoid-mediated transactivation. Cells were microinjected with an inducible CRE-lacZ, CMX-lacZ, or a natural constitutively expressed promoter of the *ras* gene driving lacZ(ras-lacZ) (a), CRBPII2-lacZ (b), βRE-lacZ (c), or MMTV-lacZ (d) reporter together with control IgG alone, antiserum to CBP, or dominant-negative peptides as indicated, and receptor-expression vectors, then stimulated with 10 μM forskolin/25 μM IBMX (CRE-lacZ) for 2 h (a), or overnight with 100 nM LGD1069 or 1 μM 9-cis-RA (b), 1 μM tRA (c), or 1 μM dexamethasone (d), respectively. Following stimulation, cells were fixed and assayed for LacZ (β-gal) activity as well as antibody content. Bar graphs summarize the percentage of cells that were positive for β-galactosidase; the number of cells injected is shown in parentheses. **METHODS.** The expression constructs CMX-lacZ, CMX-hRXRα, CMX-hRARα, RSV-hGRα have been described^{23,24}. CRE5-lacZ, and *ras*-lacZ were gifts from J. Meinkoth and K. Umesono, respectively. CRBPII2-lacZ, βREX2-lacZ, and MMTV-lacZ were constructed by inserting appropriate promoter fragments from the TK-luc reporters into pLsvn-pxln-lacZ vector (gift from S. O'Gorman). REF52 and NIH3T3 fibroblasts were cultured in 7.5% CO₂ in DMEM containing either 10% fetal bovine or 10% calf serum, respectively. Cells were plated on glass coverslips and rendered quiescent by serum deprivation in medium containing 0.05% calf serum 24–48 h before injection. DNA, antibodies and peptides were introduced at the following concentrations: CRE-lacZ, CMX-lacZ, and *ras*-lacZ (100 ng μl⁻¹); receptor-responsive reporter plasmids (200 ng μl⁻¹); receptor expression plasmids (100 ng μl⁻¹), nonspecific, affinity-purified rabbit IgG (2.5 and 5 mg ml⁻¹; Sigma); affinity-purified polyclonal rabbit antiserum to CBP (amino acids 634–648) (5 mg ml⁻¹)¹⁷, GST or GST-CBP-Δ1K3Q (3–5 mg ml⁻¹), KIX and KIX mutant (5 mg ml⁻¹)²⁰. About 10⁻¹⁵ l per cell was injected with an Eppendorf semi-automated microinjection system. Injected antisera were visualized in permeabilized cells with a secondary antibody conjugated to FITC (Jackson Immunoresearch Lab); cells containing a blue precipitate were scored as positive.

and RAR, TR-mediated transcription is also enhanced (Fig. 3d). When a CBP vector is co-transfected with either RXR/TR or TR alone activity is remarkably stimulated (compare lanes 4 and 5 or 3 and 7). Although either CBP or P300 increases basal reporter activity (lanes 9 and 10), this is fully suppressed in the presence of receptor (compare lanes 6 and 10). Interestingly, TR consistently shows a stronger ligand dependent interaction *in vitro* as well as being more responsive to CBP *in vivo*.

The necessity for CBP in receptor signalling was tested by microinjecting neutralizing polyclonal antibodies against CBP into mammalian fibroblasts. This antiserum recognizes both CBP and P300 (data not shown), and when microinjected into the nucleus, blocks transcriptional activation of a CRE-lacZ reporter gene (Fig. 4a)^{17,18}. This effect is specific, as the cytomegalovirus promoter (CMX-lacZ), as well as a constitutive cellular promoter (*ras*-lacZ) are not blocked by antiserum against CBP (Fig. 4a)¹⁷. In contrast, induction by 9-cis-retinoic acid (or LGD1069) of a CRBPII-lacZ reporter was almost completely inhibited when nuclei were injected with anti-CBP antiserum (Fig. 4b, control and anti-CBP (αCBP)). Interestingly, injection of the GST-CBP-Δ1K3Q fusion protein completely inhibits reporter activity (CBP-Δ1K3Q); GST alone has no effect. This dominant-negative effect could be due to competition of CBP-Δ1K3Q with wild-type CBP or the presence of an inhibitory function that fails to relieve repressor activity of the complex. In contrast, microinjection of a dominant-negative peptide corresponding to the CREB binding domain (KIX) or its mutant (KIX and KIX mutant) has no effect.

We next investigated the role of CBP/P300 in retinoid and glucocorticoid signalling. Injection of expression constructs for RXR and RAR results in retinoic-acid-dependent activation of a βRE-lacZ reporter (Fig. 4c, control). The reporter activity was drastically reduced with antiserum against CBP (αCBP). Surprisingly, microinjection of the KIX peptide also diminished the reporter activity, whereas a mutant of this peptide had no effect (KIX and KIX mutant). Finally, a glucocorticoid-responsive reporter (MMTV-lacZ) was tested for CBP sensitivity. Again, activation of the reporter by ligand was blocked by microinjection of CBP antiserum (Fig. 4d, control and αCBP). As with RAR (but not RXR), the glucocorticoid receptor was effectively inhibited by the dominant negative KIX peptide (KIX) but not the mutant peptide. In summary, these *in vivo* results support a role for CBP/P300 not only as cofactor for AP1 and CREB, but also as a common regulator of nuclear receptor signalling. Thus, they may be mediators of 'cross-coupling'.

The existence of other receptor cofactors (such as SRC1, RIP140, TIF1, and other associated factors) suggest that several proteins may simultaneously bind to receptors, perhaps forming an activation scaffold. The role of these individual proteins and how they contact the basal transcription machinery is unclear, but they may help the hormone response to be integrated into different physiological pathways. Because CBP/P300 associate with nuclear receptors²⁵, CREB and AP1 as well as targets of adenoviral E1A and HTLV-1-Tax²⁶ proteins, we suggest that they might be more appropriately termed cointegrators to reflect their roles in multiple regulatory circuits. □

Received 17 April; accepted 5 July 1996.

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ACKNOWLEDGEMENTS. D.C. and V.L. contributed equally to this work. We thank members of R.M.E.'s laboratory for reagents and for critically reading the manuscript, and T.-P. Yao, D. Livingston and M. Brown for communication of unpublished results. D.C. is supported by a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund for Medical Research. V.L. is supported by a post-doctoral fellowship from the American Cancer Society. I.G.S. is supported by a postdoctoral fellowship from the California division of the American Cancer Society and funds from the J. Aron foundation. R.M.E. is an Investigator of the Howard Hughes Medical Institute at the Salk Institute. This work was supported by grants from the NIH (R.M.E.), The Jane Coffin Childs Memorial Fund for Medical Research (D.C.) and The American Cancer Society (V.L.).

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CORRECTIONS

Activation of complement by an IgG molecule without a genetic hinge

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Nature **363**, 628–630 (1993)

WE reported the construction of a chimaeric mouse–human mutant IgG3 molecule, HM-1 (IgG₃231Cys232), with all hinge exons deleted, but with an introduced cysteine following Ala 231 encoded by the C_H2 exon. However, we have recently noted that the mutant that was tested had four amino acids, namely Ala-Ala-Cys-Ala, introduced after Ala 231. This mutant was also produced in the laboratory, and at some point following *in vitro* mutagenesis and sequencing there had been a switching of tubes. Our original conclusion, namely that the presence of neither a long upper hinge nor a core hinge is necessary for complement-mediated lysis (CML), is still valid. IgG₃231AlaAlaCysAla232 has an upper hinge of three amino acids, and the core hinge is substituted with a single cysteine (see Table 1 below).

IgG₃231Cys232 has recently been tested on non-reducing SDS–PAGE and found to contain mostly H–L half-molecules. It has an upper hinge of one amino acid, the core hinge is substituted with a single cysteine, and the lower hinge lacks an alanine compared to the wild-type sequence. Obviously, it lacks residues necessary for effective disulphide-bond formation. IgG₃231Cys232 has some activity in CML at high antigen density, but no activity at low antigen density. □

CORRECTION

A comparative embryological study of two ornithischian dinosaurs

J. R. Horner & D. B. Weishampel

Nature **332**, 256–257 (1988)

IN this Letter we erroneously referred an embryo in an egg to the ornithopod dinosaur *Orodromeus makelai*. Further preparation of the embryo has revealed its identity to be that of the theropod dinosaur *Troodon cf. formosus*.

The unusual teeth of *Troodon*, possessing cylindrical roots similar to ornithischians, exacerbated the identity problem in these embryos and added to an already long history of confusion¹. Additional preparation has revealed that the dentary union lacks a preentary, precluding the specimen from the Ornithischia. Shape of the humerus and configuration of the metatarsals are identical with corresponding adult and juvenile elements of *Troodon cf. formosus* (in collections of the Museum of the Rockies, Montana State University, Bozeman, Montana, USA). Absence of a fourth trochanter on the femura, originally interpreted as not having ossified until post-eclosion activity, is instead indicative of its theropod affinities. □

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Inhibition of an inwardly rectifying K⁺ channel by G-protein α -subunits

Wolfgang Schreibmayer, Carmen W. Dessauer, Dmitry Vorobiov, Alfred G. Gilman, Henry A. Lester, Norman Davidson & Nathan Dascal

Nature **380**, 624–627 (1996)

IN the Correspondence and materials section of this letter, which lists the nucleotide positions of antisense oligodeoxynucleotides (ODNs) used in this work, the nucleotide positions of anti-G₂₁₁ ODN were 546–517 (and not 918–891 as published), and for anti-G₂₁₃ ODN they were 335–306 (and not 366–335). The rest are correct. □

TABLE 1 Amino-acid sequences of the hinges of human wild-type and hinge-mutant IgG3 molecules

	Genetic hinge		Lower hinge	s-s	CML
	Upper hinge	Middle hinge			
IgG ₃	216 ELKTPGLGDTTHT		231 APELLGGP	+	+
IgG ₃ 231Cys232	A	CPRCP (EPKSCDTPPPCPRCP) ₃	PELLGGP	–	–(+)
IgG ₃ 231AlaAlaCysAla232	AAA	C	APELLGGP	+	+
m0			APELLGGP	–	–

CML activity and the presence of covalently closed H₂L₂ molecules (s-s) are indicated as plus and minus signs, respectively. m0, Hinge-deleted IgG3.